MOLECULAR ANALYSIS OF THE DRY ROT FUNGUS *Serpula lacrymans*.

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This thesis is presented to the
Council for National Academic Awards
in partial fulfilment of the
requirements for the award of the
degree of Doctor of Philosophy.

Department of Molecular and Life Sciences,
Dundee Institute of Technology.

September, 1992.

I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed [redacted] Date 18/1/93...

(Director of Studies)

Sarah, John and I

wish to dedicate this thesis to

Peter.

1971 - 1985.

Acknowledgements.

I would like to express my gratitude to my supervisors Professor Bernard King and Drs David Button and John Palfreyman for their encouragement, supervision and guidance throughout this project and for constructive criticism of this manuscript. I hope that completion of this work is adequate recompense for the debt which I undoubtedly owe them, the last named in particular, for having faith in my scientific abilities at a time when I had neither faith nor ability.

I am grateful to Professor W. Emond for enabling me to have a year's secondment in order to complete this work; and I also wish to thank my external advisor, Dr. J.A. Carey of the Building Research Establishment, for her advice.

Other members of staff at DIT who deserve thanks are Mr. George Hunter and Mr. Charles Craig, for the preparation of photographs; Miss Margot Dunnachie, for the maps and drawings; and Miss Moira Malcolm and the operations' staff of the computer centre, who guided me in the use of Mass11. Thanks must go to those who ensured that a room was always available in which to write up this thesis, viz Mr. Duncan M^{ac}Leod, other members of the janitorial staff and, especially, Miss Doris Kinnison, Canteen Manageress.

I am very appreciative of the advice, encouragement and acceptance of my fellow research students. I feel privileged to have been part of their company! Drs Heather Glancy, Elspeth Henry and Sandra Hainey, Mrs. Una Henry and other friends in many departments at DIT, especially those amongst the technical staff of MLS, will always be especially valued for their support and friendship.

Finally, special thanks are reserved for my daughter Sarah and my husband John. Whilst the former typed the references and the latter checked the references, stuck on photographs and collated this thesis, more importantly both have shared the trials and tribulations of the past seven years. Thanks you two!

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MOLECULAR ANALYSIS OF THE DRY ROT FUNGUS Serpula lacrymans.

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Abstract.

SDS-PAGE/silver staining and western blotting were used to investigate the molecular profile of a PBS soluble extract from standard mycelium of S. lacrymans FPRL 12C. Both techniques enabled a reference profile for this organism to be established. A polyclonal antiserum raised against standard mycelium of S. lacrymans FPRL 12C was used as the immunological probe.

The profiles of other isolates of S. lacrymans and different Basidiomycetes were compared with FPRL 12C. Protein and antigenic similarity indices enabled differences between these organisms to be quantified. 2 isolates of S. lacrymans differed from FPRL 12C but to a lesser extent than species in other genera. Lectin staining confirmed these observations and indicated that the majority of antigens were glycoproteins.

SDS-PAGE/silver staining and western blotting allied with similarity indices were found to aid in identification studies which established the identity of one of the anomalous isolates of S. lacrymans. Variation in basic parameters of culture caused minor changes of profile but potentially lethal factors had great effect on profiles. Antigenic differences attributable to growth phase were noted in young and aged mycelium of S. lacrymans.

Protein and antigenic profiles indicated that S. lacrymans in pine and lime sapwood blocks was more similar to young mycelium at low weight loss and to aged mycelium at high weight losses.

Protein and antigenic profiles of field samples of S. lacrymans varied markedly from those of the reference isolate. Protein profiles allied with similarity indices were only appropriate for investigation of a few field mycelial and basidiocarp samples but western blotting identified antigens in most field samples. Identification was made of antigens (a) common to S. lacrymans FPRL 12C and fresh field samples and (b) associated with either fresh or desiccated field samples. The use of these antigens for development of more specific immunological probes is discussed.

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Abbreviations.

Ab	antibody
Ab1	primary antiserum
Ab2	secondary antiserum
Ag	antigen
B	non-fat dried milk (Blotto)
BCPIP/NBT	bromochloroindolyl phosphate/nitroblue tetrazolium
BSA	bovine serum albumin
Con A	Concanavalin A
DAB	diaminobenzidine
DAB/N	diaminobenzidine enhanced with nickel chloride
DIG	digoxigenin
DMSO	dimethyl sulphoxide
ELISA	enzyme linked immunosorbent assay
HMW	high molecular weight standard proteins
HRP-Ab2	horse radish peroxidase linked secondary antiserum
IgG	immunoglobulin class G
kDa	kilo-Dalton
LMW	low molecular weight standard proteins
ME	malt extract
MEB	malt extract broth
MW	molecular weight
NCS	newborn calf serum
NDS	normal donkey serum
NGS	normal goat serum
NP 40	Nonidet P40
NRS	normal rabbit serum
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline, 10mM, pH 7.4
PMSF	phenylmethyl sulphonyl fluoride
rpm	revolutions per minute
R/T	room temperature
Rf	relative mobility
SDS	sodium dodecyl sulphate
T	Tween 20
TEMED	N,N,N',N'-tetramethyl-ethylene diamine
TLS	tangential longitudinal section
TN	total nitrogen
u-p	ultra-pure
WGA	wheat germ agglutinin

CHAPTER 1. INTRODUCTION.

1.1 Timber and decay.

1.1.1 The importance of wood.

Wood has always been of the utmost importance to man. Archaeological records indicate that wood has been used as fuel and for construction purposes since prehistoric times. It has been essential not only for the building of houses and defensive sites but also for the manufacture of carts, ploughs, weaponry and boats. Despite the advent of the use of metals, stone and concrete for many of its former purposes, wood retains its pre-eminence in the modern world as a vitally important industrial raw material throughout the Third World and industrialised countries. In solid form it is used for construction purposes for houses and boats, and also for fuel, furniture and even jewellery, whilst in composite form it is used to make paper, plywood and fibreboard. Indeed, it is hard to imagine the world of the 1990's without wood.

1.1.2 World requirements of wood.

The total world consumption of roundwood, i.e. timber as felled with no processing, in 1988 was 3,327 million cubic metres (m^3) (Geographical Digest, 1990). 54% was used for fuel wood, only 7% of which was used by the developed countries. The industrialised nations used 35% of the remaining 46% of world wood consumption for industrial purposes, with the remainder being used for this purpose by Third World countries. In 1988 worldwide production of non-coniferous roundwood was 1970 million m^3, with India producing the largest volume (234 million m^3) and the UK near the bottom of world rankings (0.8 million m^3). In the same year the world production of coniferous roundwood was

1357 million m^3; the USA led world production figures (342 million m^3) whilst UK production was only 4.3 million m^3. Production of sawnwood, i.e. timber in the first stage of processing (in rectangular form), mirrors a country's production of roundwood (Geographical Digest, 1990).

Wood and wood products are of great economic importance in world trade since highly populated industrialised small countries cannot produce enough for their own needs and they must import their requirements. For example, solid wood and wood products imported into the UK in 1988 amounted to £1356 million, of which £245 million, £307 million and £184 million worth came from Sweden, Canada and Finland respectively (Geographical Digest, 1990). The importance that wood based industries can have to a country's economy can be illustrated by Sweden, which is a small country, but which is the world's third largest exporter of timber products such as sawn timber, wood pulp, cellulose, packaging materials, newsprint and special papers. These forest based industries employ only 6% of the work force, 250,000 people, yet they produce 20% of the monetary value of Sweden's exports (Marsden & Marsden, 1989).

1.1.3 Source of wood.

Wood is the strengthening and supporting material found in, and produced as a secondary tissue by, the stem of a tree. Stems typically serve as an aerial mechanical support for leaves, flowers and fruit and, additionally, provide a path of conduction between these organs and the soil-bound roots. Trees are mature individuals of the seed plants, the Spermatophyta, and are differentiated from shrubs by their greater height and the possession of a single woody trunk. The Gymnosperms and the Angiosperms are the two principal groups within the seed plants and both groups contain wood

producing species (Hill et al., 1960) which when mature will form a climax vegetation of commercially exploitable forest. More than half the world's forests are composed of Angiosperm species (Myers, 1985). In 1986 31% of the total land area of the world was covered by forests and, whilst as much as 76% of Finland and 50% of Sweden was tree covered, only 9.5% of the UK was similarly clad (Geographical Digest, 1990).

The Gymnosperms produce 'naked' seeds in cones, hence the name given to the trees within this group - the conifers. They are widely distributed, being found in regions with Mediterranean and temperate climates as far north as latitude 70^0, and include species such as the pines, spruces, larches, hemlocks and cedars. The USSR contains 60% of the world's coniferous forests, with a further 25% in N. America (Myers, 1985). The Angiosperms ('hidden' seeds) are further subdivided into two groups; of which the monocotyledons, the grasses, do not produce woody tissues. The second group, the dicotyledons, contains some members which produce wood. The broad leaved trees, e.g. lime, oak, beech, sycamore and eucalyptus, are all dicotyledons. They have natural worldwide distribution from equatorial regions, through temperate zones to the tundra (Hill et al., 1960) but the commercially important stands of broad leaved trees are found within the range of latitude from 50^0N to 45^0S (Readman & Mayers, 1986).

1.1.4 Commercial wood.

Wood obtained from Angiosperms is commonly termed hardwood and that harvested from conifers is termed softwood. However, the density and hardness of a particular wood is largely governed by the proportion of thick walled cells present and the terms 'hardwood' and 'softwood' do not

always accurately describe the strength or consistency of wood. Indeed balsa wood, (Ochroma sp.), is the softest wood but it comes from a dicotyledon and is technically a hardwood. The terms hard and softwood derive from the medieval timber trade (Butterfield & Meylan, 1980) and were coined because the commercially important tropical dicotyledons normally produce a harder timber than the temperate conifers. In temperate countries softwoods are the most important type of timber used for constructional purposes (Coggins, 1980). Hard woods have traditionally been used for joinery and floors which are to undergo heavy wear but they are also used in situations where their greater decorative properties can be appreciated.

1.1.5 Wood for construction.

Wood gives organisms the potential to reach great vertical heights in an aerial environment; for example, the Californian redwood (Sequoia sempervirens) which is native to the west coast of the USA can reach a height of 110m but such heights are exceptional and rivalled only by some of the Australian eucalypts. In Britain, mature specimens of Scots pine (Pinus sylvestris), larch (Larix decidua) and beech (Fagus sylvatica) can all attain heights of 40m (Clapham & Nicholson, 1975). Man has capitalised upon the strength and supporting function of wood by using it for construction purposes. It is an ideal building material since it is, weight for weight, stronger than steel; relatively lightweight in comparison with concrete or steel; aesthetically pleasing and, in contrast to the finite reserves of iron or plastic based compounds, wood stocks are renewable when given proper management. The inherent disadvantages of wood have been largely overcome by modern

technology, e.g. lamination processes have overcome the natural size limitations of wood and modern fire retardants limit the extent to which wood can be destroyed by fire (King, 1981).

1.1.6 Wood anatomy.

A cross-section of a trunk shows two well defined regions - a relatively thin external bark surrounding a cylinder of secondary wood which can be 1m or more in diameter. These two regions are separated by a layer of meristematic tissue, the vascular cambium, which is only a few cells thick. This meristem is responsible for the development of all secondary tissues which contribute to the growth in diameter of the trunk. Wood is composed of secondary xylem cells (tracheids and vessels) and living axial and ray parenchyma cells and fibres. The annual production of wood over successive years creates the trunk of a mature tree which is mainly composed of concentric cylinders of wood, annual rings, with an insignificant amount of primary xylem and pith in the centre (Hill et al., 1960).

The outermost 10 - 12 annual rings are composed of wood which is light in colour and is called sapwood. By definition (Butterfield & Meylan, 1980) sapwood is secondary xylem where the tracheary cells are active in water conduction and the physiological activities of the wood are performed by living axial and ray parenchyma cells and fibres. However, most water is transported up the trunk by the wood in the two or three annual rings nearest the cambium. As the wood ages the cells in the older annual rings nearer the centre of the trunk become progressively lignified, and therefore strengthened, and darker because of deposition of extractives and other extraneous materials in

Figure 1: Simplified diagrams of longitudinal sections through secondary xylem elements (adapted from Jane, 1956).

Figure 1a): Gymnosperm tracheid.

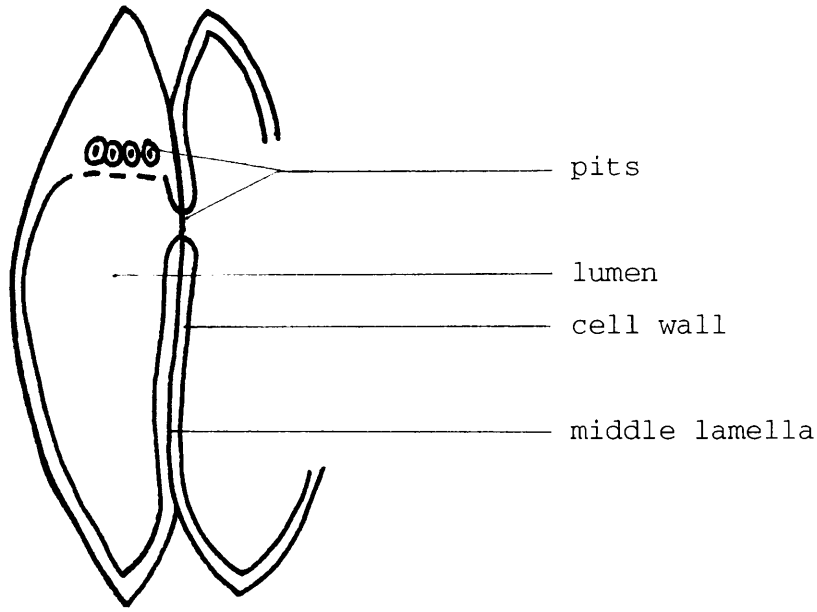
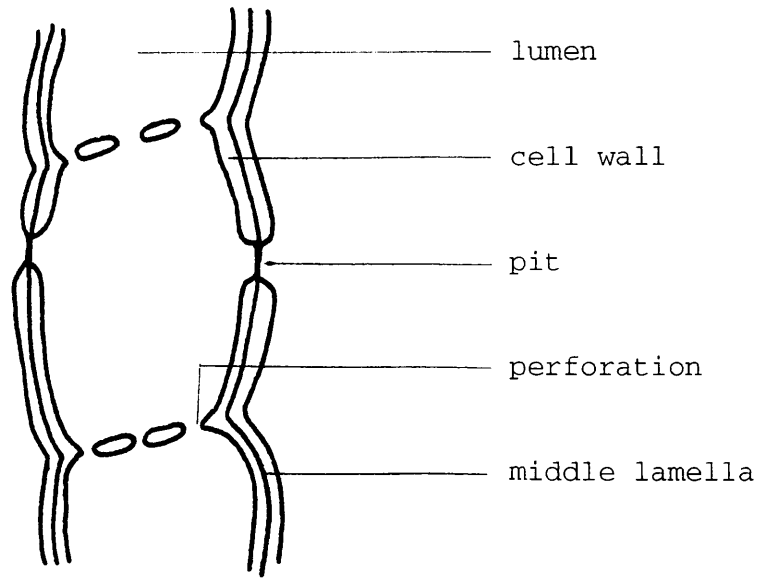


Figure 1b): Angiosperm vessel element.



the cells. This wood is called heartwood and is a physiologically dead tissue which provides the chief support for the stem of a mature tree. Heartwood is usually more durable in usage than sapwood and is commercially more valuable than sapwood.

1.1.7 Wood microstructure.

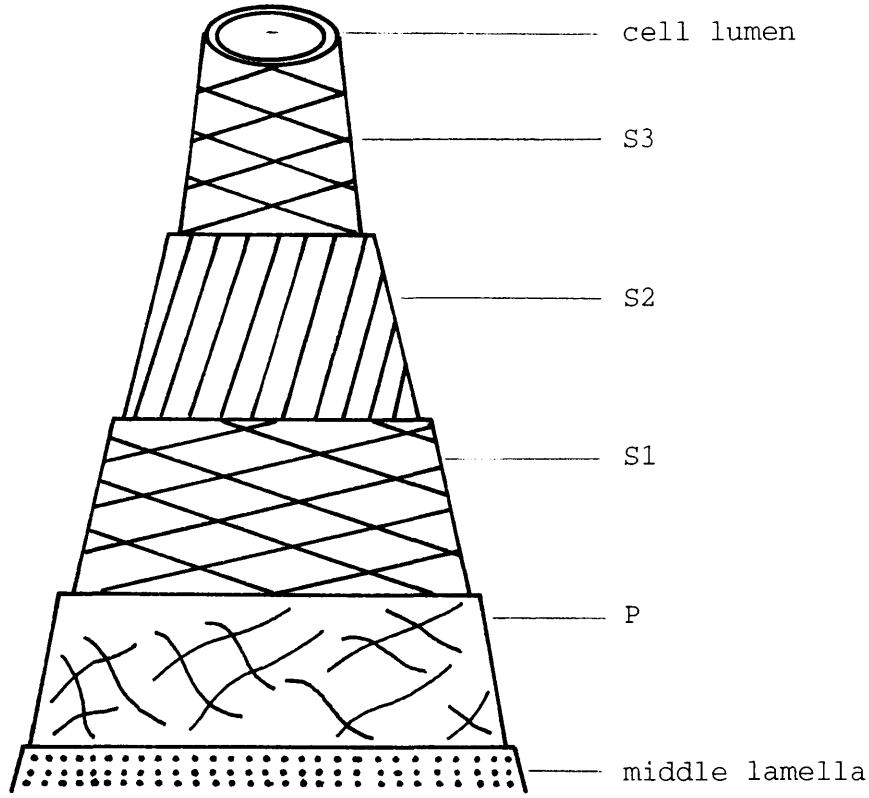
The microstructure of wood varies greatly between species and particularly between hard and softwoods. Softwoods are fairly uniform in texture and relatively easy to work with because 95% of the wood volume of coniferous species may be composed of longitudinal tracheids (Figure 1a). These are axially elongated cells which can be up to 10 mm in length, with walls of varying thickness and pointed ends which are densely intertwined with adjacent cells (Butterfield & Meylan, 1980). They function in support and conduction; the passage of solutes between tracheids is via opposing pairs of pits which pierce the secondary walls of contiguous tracheids.

The distinctive cell type in hardwood species is the vessel element (Figure 1b). These are relatively thin walled tubular, open ended cells which, when joined end to end, form the xylem vessel. In common with tracheids they have no living contents at functional maturity. These vessels are specialised for longitudinal water conduction in the tree (Kollman & Cote, 1968) since pits on their side walls and perforations between the end walls of vessel elements facilitate unimpeded upward flow to water and solutes.

1.1.8 Wood cell walls.

An overall pattern is common to all wood cells in that each is surrounded (Figure 2) by a layer known as the middle lamella within which is a primary wall (P) and a

Figure 2: Simplified structure of wood cell wall showing the angle of orientation of the microfibrils in each of the major wall layers (adapted from Desch & Dinwoodie, 1981).



Key: S1, S2 and S3 represent the secondary wall layers. P represents the primary wall of the wood cell.

thick secondary wall consisting of 3 layers (S1, S2 and S3); another layer (warty layer) may additionally be deposited spirally on the luminal side of the S3 layer. The S2 layer can make up 50% or more of the total wall thickness (Butterfield & Meylan, 1980).

The walls of wood cells are chiefly composed of cellulose, hemicelluloses and lignin. Holocellulose is a term applied to the polysaccharide, lignin-free, fibrous material making up the cell wall. The wood cell wall is a composite containing 'fibre' and 'matrix' components (Desch & Dinwoodie, 1981). Cellulose molecules lying parallel to each other form crystalline cellulose which comprises the 'fibre' component whose function is to confer tensile strength. Non-crystalline cellulose, hemicelluloses and lignin are the components which form the matrix. Their combined function is to facilitate upright growth in an aerial environment by conferring a degree of elasticity combined with rigidity to the matrix. Non-crystalline cellulose is formed at intervals along the length of the crystalline cellulose molecule where the parallel nature of the molecules is broken down. Lignin is chiefly responsible for rigidity and the hemicelluloses possibly form a primary matrix upon which lignification occurs. The cellulose, hemicellulose and lignin form a unit called a microfibril which is of indeterminate length and 10 x 5 nm in cross-section (Desch & Dinwoodie, 1981). The subdivisions of the wood cell wall are differentiated by the internal arrangement of these microfibrils (Figure 2).

The relative proportions of cellulose, hemicellulose and lignin can vary according to the wood type. Temperate zone hardwoods generally contain 17-24% lignin (per total weight of wood) whereas conifers usually vary between 25-34% lignin. The cellulose content of most temperate zone woods,

both hard and softwoods, varies between 40-50%. Hemicelluloses comprise the remainder (Kirk, 1984). The species of tree within a wood type can affect the proportion of these constituents. Betula papyrifera (paperbark birch), an Angiosperm, was found to contain 42% cellulose, 19% lignin and 38% hemicellulose whereas the proportion of these in another Angiosperm, Ulmus americana (American elm), was 51%, 24% and 23% respectively. This variation can also be seen within the Gymnosperms since Pinus strobus (Weymouth pine) was found to contain 41% cellulose, 29% lignin and 27% hemicelluloses but the proportions of these in Tsuga canadensis (Canadian hemlock) were 41%, 33% and 23% respectively (Kirk, 1984).

1.1.9 Durability of wood.

Whilst the middle lamella and the primary wall are strongly lignified this chemical is relatively less abundant in the secondary wall (Jane, 1956). Lignin is a material which is hard to remove from wood, even in the laboratory situation, and its presence contributes to the durability of wood (Kollman & Cote, 1968). Durability, in relation to timber, may be defined as the ability of a material or object to endure (Cartwright & Findlay, 1958). The durability of a timber includes its resistance to fungal decay, to insect attack, to mechanical wear and to the destructive effects of exposure to weathering agents such as frost, sun and sandstorms. Timber which is protected from these destructive forces will last indefinitely since timber does not deteriorate by age alone; indeed, the wooden objects which are found in museums are testament to the inherent durability of wood. Durable timbers in commercial use include the hardwoods afrormosia, jarra and teak; and

the softwoods yew, American pitch pine and western red cedar (Cartwright & Findlay, 1958).

Durability is conferred not just by the presence of lignin but also by other substances deposited in the cell walls of heartwood which can be extracted by solvents and which are consequently known as extractives. Examples of these are resins, which are formed in the softwoods, and tannins, which are formed in the oak and chestnut (Wilkinson, 1979). Many of these extractives are toxic to fungi and there have been many attempts to link resistance to fungal decay and durability to the extractives in a particular timber. In practice it is observed that sapwood of all species is readily decayed by fungi and it is only the heartwood of durable species which is resistant to fungal decay. This helps to confirm that it is the presence of the extractives which makes timbers resistant to decay and which contributes to durability.

1.1.10 The polymeric nature of the wood cell.

The major components of the wood cell wall are all natural polymers. Cellulose is a polysaccharide, a polymer of glucose. The various hemicelluloses are also polysaccharides but they differ from cellulose in that they contain arabinose, mannose and xylose, as well as glucose (Wilkinson, 1979). Lignin, though also a polymer, is not a polysaccharide but is derived from phenylalanine. It is synthesised via coniferyl alcohol (Gymnosperms) or coniferyl and sinaptyl alcohols (Angiosperms) via a complex process which is not fully understood (Kirk, 1984). There are also small amounts of protein in wood, the nitrogen content of wood is usually stated as ranging from 0.03 - 0.127% of the dry weight of the wood (Deacon, 1984).

Wood contains many other substances, including starch and inorganic silica, in addition to the major components, extractives and protein. Overall wood is a material which contains a rich pool of organic polymers which have great potential as a source of nutrients for other biological organisms. A wide range of organisms, in particular the ascomycete and basidiomycete fungi and the Fungi Imperfecti, are able to exploit these nutrients.

1.1.11 Tree pathogens.

Both living and harvested wood material is susceptible to decomposition but the living tree has an inherent resistance to most biological degradation due to the presence of a variety of defence mechanisms. When the bark of trees is wounded the cork cambium forms a callus tissue which is a corky tissue made up of large, thin walled cells which protect the injury from invasion by pathogens (Hill et al., 1960). Plants produce chemicals which can inhibit destructive organisms, e.g. secondary metabolites which deter feeding by herbivores are common (Palo, 1984); and the wood of the goat willow (great sallow), Salix caprea, produces flavenoids which may be of relevance in the damp habitat of this willow since they have been shown to act as inhibitors of some wood destroying fungi (Malterud et al., 1985). However, just as different wood types and species vary in their durability so different living tree species vary in their susceptibility to decay.

There are a number of parasitic fungi which attack living trees and some of these pathogens have important economic implications. Dutch Elm Disease has decimated elm (Ulmus spp) populations in North America and Eurasia and is caused by the aggressive strain of the ascomycete fungus Ophiostoma (Ceratocystis) ulmi. The elm bark beetles

Scolytus scolytus, S. laevis and S. multistriatus act as vectors for the fungal spores which enter xylem vessels of elm as a result of the beetles feeding on twigs in the crown of the tree. Germination results in the vegetative mycelial phase of growth in the xylem, conidia spread rapidly through the tree with the sap flow and heavily infected trees die within a few years as a result of widespread blockage of xylem vessels (Brasier, 1984). Heterobasidion annosum and Armillaria mellea are two basidiomycete fungi which are also of economic importance in living trees; H. annosum invades and kills the root tissues of coniferous trees, whilst A. mellea is a pathogen of both conifers and Angiosperms. Both organisms cause root rot and death of saplings and both can cause decay of commercially valuable heartwood in the adult tree (Hudson, 1986).

1.1.12 Biodegradation of wood.

At the end of the life of a woody tree, i.e. either when it dies naturally or when it is felled for commercial use, the constituents of wood would naturally be slowly returned to the ecosystem by decomposition brought about by a variety of decay organisms. This environmentally beneficial process of degradation increases the humus content of soil and, via the processes of the carbon and nitrogen cycles, recycles minerals and releases carbon dioxide to the atmosphere, thereby making them available for use in the metabolic processes of other organisms.

The fungi are of prime importance in the biodegradation of wood. However, in conjunction with the biodegradation of wood by fungi there are a number of types of animal which will decompose timber. In an aerial environment these include insects such as termites, in the tropics, or wood worm beetles, in temperate regions; whilst molluscs such as

the marine Teredo are responsible for a great deal of damage in aquatic environments. Wood which is used by man for structural and functional purposes is subjected to a further type of degradation, viz mechanical wear and chemical stress. A review of all these types of decomposition can be found in Cartwright & Findlay (1958).

1.1.13 Biodeterioration.

Recognition that the decay of wood is primarily caused by fungi is generally attributed to Robert Hartig whose work was published in 1878. However, in an opinion far in advance of scientific knowledge of the time (Findlay, 1974), Benjamin Johnson of Ipswich noted the close association between the dry rot fungus and the decayed state of wood and stated in a letter in the Transactions of the Royal Society of Arts in 1803 that the rot 'was due to a visit from a plant'.

Whilst biodegradation implies saprophytic activity of fungi which has beneficial effects for the biosphere (Hill et al., 1960) it is self evident that the decay of wood which is harvested by man is undesirable. A great deal of money is therefore spent on prevention of degradation of commercial wood by micro-organisms. This type of degradation which consists of biological breakdown with adverse implications for man, either on economic grounds or on the grounds of health and safety, has been termed biodeterioration (Hueck, 1965).

Timber which is at, or below, 20% moisture (weight for weight) is immune to fungal attack but above this level the risk is high, especially above the fibre saturation point, approximately 30% of the initial oven dry weight of the wood. Optimal moisture levels for the decay of timber are usually stated to be in the range 30 - 40% depending on the

species of fungus (Coggins, 1980) but this may be a generalisation since the range for C. puteana extends from 30 - 70% (McDowell, personal communication). The most favourable condition for the growth of fungi in wood is when the cell walls are fully imbibed and are additionally coated with a film of liquid water but where air spaces remain in cavities so that gaseous diffusion can occur. On hydration, wood polymers swell which creates openings, microfibrillar spaces, in the polymer matrix which do not exist in dry wood. The swelling brought about by hydration also enlarges the existing pits and perforations.

1.1.14 Fungal hyphae.

In filamentous fungi the morphological form which is responsible for invading and exploiting the substratum is the vegetative hypha. This is essentially a tube consisting of a rigid cell wall surrounding protoplasm which is sub-divided into interconnecting compartments. It usually has a consistent diameter ranging from 1 - 30 µm. The apical compartment (150 - 500 µm long) has a tapered extension zone at its tip (up to 30 µm long) which represents the region of most active wall growth. Inside the cytoplasm of the extension zone is a marked accumulation of membrane bound vesicles which play a major role in growth (Deacon, 1984). Progressive ageing is seen in the compartments behind the apex as vacuoles become progressively larger until the cytoplasm and nucleus is constricted to a thin peripheral zone. In the oldest part of the hypha the cytoplasm and wall may break down by autolysis.

The cell wall is a dynamic, multilayered structure which determines the tubular shape of the cell. The range of differentiated structures associated with an organism are a direct result of the wall components. Additionally, the wall

acts as an interface between the fungus and the environment; it protects the cell from lysis; it acts as a binding site for some enzymes and it has antigenic properties which mediate the interactions of fungi with other organisms (Deacon, 1984).

1.1.15 Fungal cell walls.

Cell walls of filamentous fungi are usually surrounded by a layer of extra-hyphal mucilage. The walls of all fungi contain crystalline 'fibrillar' and amorphous 'matrix' components but their chemical composition varies between taxonomic classes and morphological forms of the same species (Hunsley & Burnett, 1970; Garcia-Mendoza, Avellan & Novaes-Lediev, 1987; Bartnicki-Garcia, 1968). The fibrillar component in the walls of brown rot fungi are microfibrils composed of a polysaccharide, chitin, which is a straight chain β-(1-4)-linked polymer of N-acetylglucosamine. The matrix components are also polymeric and include various glucans, mannans and proteins. There are 2 major types of glucans recognised (Deacon, 1984):

a) R-glucans which represent 15 - 30% of the wall polysaccharide and are branched chains with β-(1-3)-backbone and some β-(1-6)-linkages. These probably form cross-linkages with other wall components and strengthen the wall.

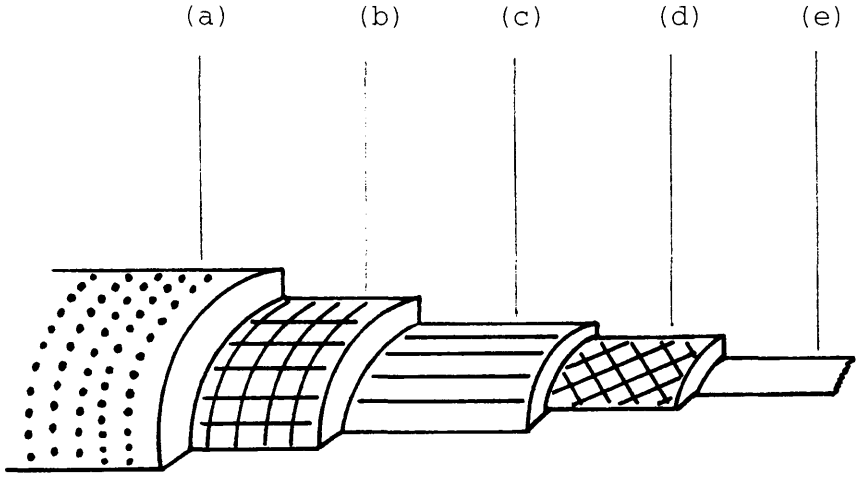
b) S-glucans which represent 15 - 30% of the wall polysaccharide and are composed of α-(1-3)-linkages.

In Schizophyllum commune, a white rot Basidiomycete, the cell wall is composed of 10% chitin, 61% glucans, 7% protein, 3% lipid and <3% mannans (Hunsley & Burnett, 1970).

1.1.16 Wall architecture.

Early studies of fungal surface compounds concentrated on the chemical nature of well washed mycelial walls (Taylor

Figure 3: Diagram to illustrate the wall architecture of a mature hypha of Neurospora crassa based on studies with sequential enzymic treatments (adapted from Hunsley & Burnett, 1970; Burnett, 1979).



Key: (a) outermost layer of amorphous glucans, c. 87nm thick.

(b) glycoprotein network embedded in protein, c. 49nm thick.

(c) protein, c. 9nm thick.

(d) Randomly arranged chitin microfibrils embedded in protein, c. 18nm thick.

(e) plasmalemma.

& Cameron, 1973). Hunsley & Burnett (1970) sequentially dissected the hyphal walls of S. commune and Neurospora crassa with enzymes. Subsequent electron microscopy revealed a wall approximately 160 nm thick composed of 4 structural regions which graded into each other (Figure 3). These were

a) an outermost layer of amorphous glucans with predominantly β-(1-3) and β-(1-6) linkages;

b) a network of glycoprotein embedded in protein;

c) a layer of discrete protein and

d) an innermost layer of chitin microfibrils embedded in protein next to the plasmalemma.

At the hyphal apex the wall is thinner (c. 50 nm) with only an innermost layer of chitin embedded in protein and an outer layer composed mainly of protein. The bulk of the hyphal wall is laid down in the apical 1 μm dome. Growth at the apex involves deposition of new wall material and is thought to involve a delicate balance between synchronous wall lysis and synthesis but the mechanism is not well understood.

1.1.17 Cell wall biochemistry.

Non-structural cell wall proteins and glycoproteins are found loosely attached to the outside of the walls and in the extra-hyphal sheath. These proteins and glycoproteins are responsible for the interaction of the hypha with the environment. Numerous enzymes, particularly hydrolases e.g. acid phosphatase, α-amylase and proteases, have been located in the cell wall (Pugh & Cawson, 1977). There is a correlation between enzyme distribution and morphology in filamentous fungi (Pugh & Cawson, 1977). In Mucor and Aspergillus the enzyme content of young hyphae is greatest at the growing tips and enzymes are secreted

extracellularly; behind the growing point the peripheral enzyme content lessens (Pugh & Cawson, 1977).

Little is known about how these enzymes cross the cell wall and little is also known at a molecular level about either the outermost surfaces of fungal hyphae or the processes involved in cell recognition and interaction which must take place at, or near, the surfaces of hyphae (MacDonald, Dunstan & Dewey, 1989).

1.1.18 Decay fungi.

A variety of fungi are responsible for the biodeterioration of wood and these can be separated into 2 categories, viz the staining and mould fungi or the wood rotting fungi (Bravery et al., 1987). Members of the former belong to 2 fungal classes, the Ascomycetes and the Fungi Imperfecti (Deuteromycetes). These spoil the appearance of the wood and consequently reduce the value of the wood but there are no significant weight or strength losses in wood colonised by these fungi. The wood rotting fungi which are primarily responsible for causing significant structural damage to wood are further sub-divided into the soft rot fungi and the Basidiomycetes. The former sub-division includes members of the Ascomycetes and the Fungi Imperfecti; these produce cellulases which soften the outer layers of wood and led Savory (1954) to call them the soft rot fungi. These organisms will only colonise wood with a higher water content (>50%) than that which favours colonisation by Basidiomycetes (King, 1981).

1.1.19 The Basidiomycetes.

The Basidiomycetes require a minimum level of 20% moisture in wood for colonisation but in general moisture contents of 30 - 50% allow growth (Cartwright & Findlay,

1958). The class Basidiomycetes contains about 20% of the classified species of fungi and is further sub-divided into the Heterobasidiomycetes and the Homobasidiomycetes (Hill et al., 1960); only the latter group contains the wood rotting fungi. They are characterised by conspicuous fruiting bodies and a mycelium which frequently has clamp connections. Although Homobasidiomycetes can affect the living tree, e.g. H. annosum, most of them are saprophytic.

Wood has been described as a series of holes surrounded by nutrients (Anon.). The Basidiomycetes are able to physically and chemically penetrate these holes and, therefore, are superbly adapted to exploit the potential of wood to supply their nutritional requirements. Physical penetration of wood is aided by the tubular form of the mycelium. The hyphae within the substrate are more branched and much narrower (1 - 2 μm) (Cartwright & Findlay, 1958) than the aerial ones whose diameter can range from 1 - 30 μm (Deacon, 1984) so that the natural openings provided by pits and perforations in water swollen wood are large enough, 0.2 - 15 μm (Jane, 1956), to permit mycelial access into the wood cell lumen. Hyphae lie against the inner surface of the secondary cell wall (Figure 4) and secreted enzymes leave the fungal cell at the site of active growth, the tip. Enzyme penetration into wood is aided by the aqueous medium created by the hydration of wood which allows diffusion of enzymes and acts as a continuum between organism and substrate. However, the highly structured crystalline nature of the wood cell wall means that enzymes, e.g. cellulolytic enzymes whose average diameter is 5 nm, are unable to penetrate into the inter-microfibrillar spaces (0.5 - 4 nm at fibre saturation point (Montgomery,1982)) of the cell walls. The problem of accessibility of enzymes must be overcome, presumably through some mechanism of enlargement

Figure 4: Diagram of wood cells in transverse section comparing the pattern of decay by brown rot and white rot basidiomycete fungi (adapted from Montgomery, 1982).

Figure 4a): White rot Basidiomycete decay.

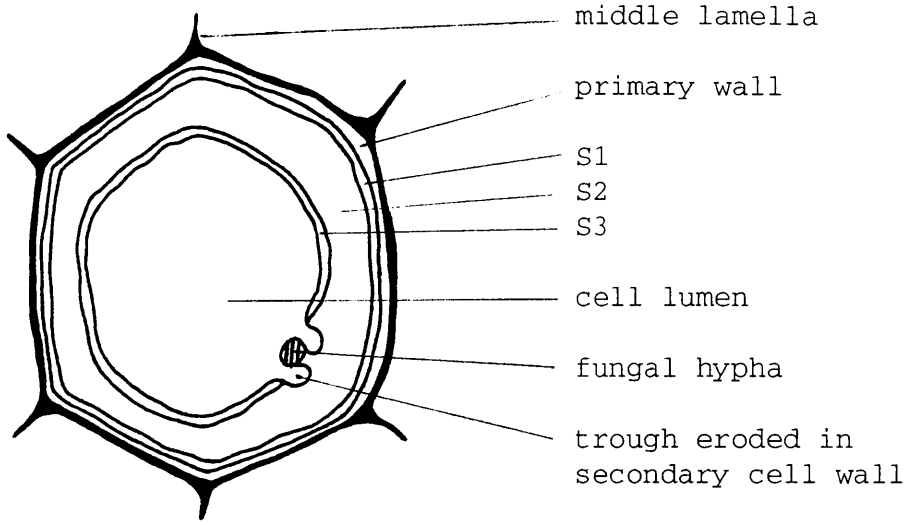
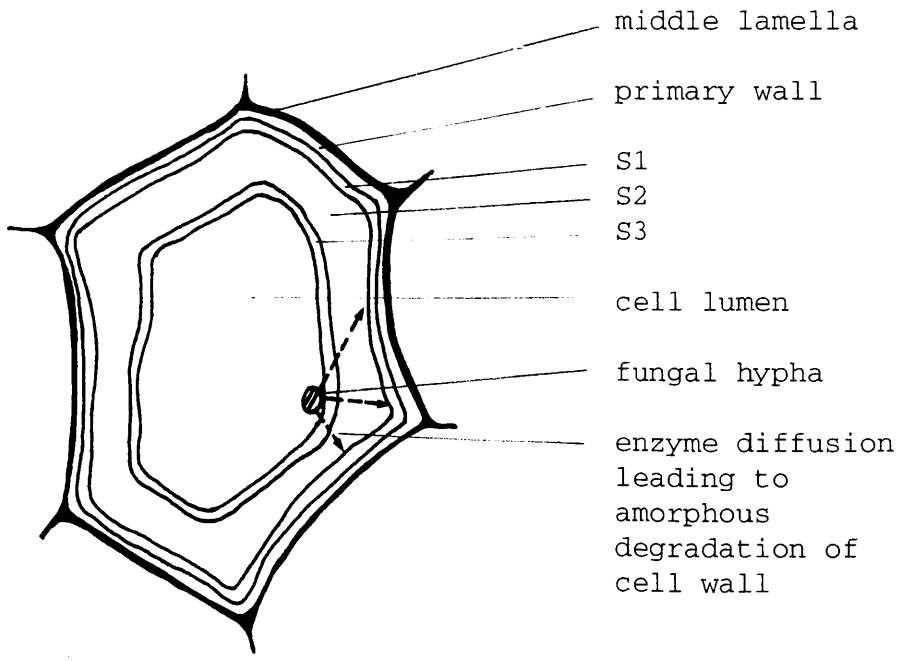


Figure 4b): Brown rot Basidiomycete decay.



of the openings (Kirk, 1973) and by non-enzymatic degradative mechanisms, to allow degradation to occur.

The wood rotting Basidiomycetes contain 2 groups, the white and brown rot fungi but this is not a taxonomic sub-division. Instead, it is based on the fact that white and brown rot fungi have developed different strategies for decaying wood and, in particular, for dealing with the lignin which serves as a chemical and physical barrier to enzymic degradation of wood polysaccharides (Kirk, 1971).

1.1.20 White rot fungi.

The white rot fungi produce ligninases and cellulases whose attack is local to the hypha (Figure 4a) resulting in complete metabolism of the holocellulose and lignin components of the cell wall. The remaining wood is white in appearance and splinters as decay proceeds. Some examples of saprophytic white rot fungi which are of economic importance in Britain are Stereum hirsutum and C. versicolor. Understanding of the specific reactions that comprise lignin biodeterioration in white rot fungi is far from complete (Eriksson, Blanchette & Ander, 1991). Evidence is accumulating that white rot fungi have a complex ligninolytic system and that enzymes move into the secondary wall and degrade the lignin that encrusts the cellulose fibrils (Cowling, 1961; Eriksson, 1985). However, work has been restricted to elucidation of chemical and physical characterisation of partially degraded lignin isolated and purified from rotted wood. Removal of the lignin exposes the polysaccharide elements of the cell wall so progressive enzymatic degradation of these components can occur.

1.1.21 Degradation of cellulose by white rot fungi.

The white rot fungi cause a gradual decrease in the degree of polymerisation of the cellulose as degradation proceeds (Kirk, 1973). Most studies on the mechanism of action of polysaccharidases have been carried out on cellulose since this is the main component of wood. There are 2 main types of hydrolytic cellulases, exoglucanases and endoglucanases, which act synergistically. The current concept of cellulose degradation is that endoglucanases act randomly within the crystalline cellulose chain, breaking chemical bonds and creating free ends to which the exoglucanases can bind prior to their hydrolysis of glucose or cellobiose units from the ends of the chain (Montgomery, 1982). To date the following enzymes from Sporotrichum pulverulentum (Phanerochaete chrysosporium), used as a model fungus, have been extensively researched (Eriksson, Blanchette & Anders, 1991):

a) five endo-1,4-β-glucanases that attack the chain at random hydrolysing the 1,4-β-glucosidic linkages (Eriksson & Pettersson, 1975);

b) one exo-1,4-β-glucanase that splits off either cellulobiose or glucose from the non-reducing end of the cellulose (Eriksson & Wood, 1985);

c) two 1,4-β-glucosidases that hydrolyse cellobiose and other water soluble cellodextrins to glucose (Eriksson & Wood, 1985).

Oxidative enzymes are also involved in cellulose degradation (Eriksson, Blanchette & Ander, 1991). To date only cellobiose dehydrogenase (Westermark & Eriksson, 1975) and cellobiose oxidase (Ayers & Eriksson, 1982) have been identified in P. chrysosporium.

1.1.22 Brown rot fungi.

Brown rot fungi cause a generalised diffuse rot resulting in wood cracking along lines of weakness into cuboidal pieces. Nilsson (1985) defined brown rot as a form of fungal decay caused by Basidiomycetes that mainly degrades the polysaccharides through extensive depolymerisation. Although some degradation of lignin is apparent the residual substance after advanced brown rot decomposition consists of an amorphous brown crumbly residue that is composed primarily of lignin. Some examples of brown rot fungi which are economically important in Britain are Serpula lacrymans, Coniophora puteana and Fibroporia vaillantii. Their hyphae produce cellulases which diffuse into the S2 layer of the wall causing rot distant from the hypha (Figure 4b). Since this diffusion would be hindered by the physical presence of lignin the hyphae also produce chemicals which affect the structure of lignin. However, brown rot organisms lack the complex enzymatic components of the ligninolytic system of the white rot fungi. The attack on lignin seems to be limited to a removal of part of the methoxyl groups (Ander & Eriksson, 1978) with the result that lignin is only modified and slightly depleted during brown rot fungal attack. Brown rot fungi strongly decrease the methoxyl content of lignin in wood (Kirk, 1975; Fukuda & Haraguchi, 1985). Aromatic hydroxyl groups are formed during this demethylation and new hydroxyl groups may also be introduced by direct hydroxylation of aromatic rings ortho to propanoid side chains (Kirk, 1975). In addition, there is also an increase in the content of oxygen due to conjugated carbonyl and carboxyl groups. However, the mechanisms by which these changes are effected have still to be elucidated (Eriksson, Blanchette & Ander, 1991).

1.1.23 Brown rot fungal decay of cellulose.

Recent observations suggest that brown rot Basidiomycetes degrade polysaccharides by a mechanism different from that operating in S. pulverulentum (Eriksson, Blanchette & Ander, 1991) since they produce endo-1,4-β-glucanases but seem to lack the exo-1,4-β-glucanases (Highley, 1975). Therefore brown rot fungi cannot degrade crystalline cellulose by the synergistic action of endo- and exo- glucanases. No other enzyme systems are known to substitute for the synergistic endo-exo-glucanase co-operation (Eriksson, Blanchette & Ander, 1991). Additionally, brown rot fungi do not appear to have the full complement of oxidative enzymes possessed by white rot fungi for cellulose degradation (Ander & Eriksson, 1978).

The main feature of brown rot decay is the rapid depolymerisation of the holocellulose, an ability apparently unique to the brown rot fungi (Eriksson, Blanchette & Ander, 1991) since white rot fungi depolymerise cellulose more slowly and utilise the degradation products simultaneously. This implies that the depolymerising agent might be of low molecular weight and can easily diffuse through the wood fibre wall to gain access to the cellulose chains. Koenigs (1974a) found that brown rot fungi are powerful producers of H_2O_2 and suggested that these fungi oxidise cellulose, with the initial attack on crystalline cellulose via an H_2O_2/Fe^{2+} system (Koenigs, 1974b). It has been estimated that there is enough iron in the ferric form in wood to sustain its decomposition but this would have to be converted to the active Fe^{2+} form (Montgomery, 1982). The H_2O_2 could be produced by the oxidation of sugars to sugar lactones by fungal enzymes and the need for this reaction would explain the findings of Highley (1978) which indicated that Poria placenta was unable to degrade cellulose unless other

polysaccharides (hemicelluloses) were present. He concluded that there was strong evidence that brown rot fungi use a non-protein oxidative mechanism to initiate cellulose breakdown in wood prior to its enzymic degradation. This was confirmed by Cobb (1982) who demonstrated that a non-enzymic cellulose decay mechanism exists in brown rot fungi. Much work remains to be done until the exact mechanisms can be elucidated (Eriksson, Blanchette & Ander, 1991).

If a H_2O_2/Fe^{2+} cellulose degrading system exists in brown rot fungi a suitable safety mechanism must also have evolved since H_2O_2 is highly toxic to living cells; it forms free radicals by homolytic cleavage that rapidly attack lipid membranes (Montgomery, 1982). The localisation of polysaccharide degradation at some distance from the brown rot hypha may well be a protective mechanism.

Respiration in fungi is similar to that in other aerobic organisms but brown rot Basidiomycetes accumulate oxalic acid in cultures. It is synthesised during aerobic respiration by oxidation of glycoxylate (Griffin, 1981). Oxalic acid creates an acid environment in wood and Bech-Andersen (1987) has argued that oxalic acid, by hydrolysis of hemicelluloses, acts as an agent for opening up the wood so that cellulose is more accessible to enzymic attack.

1.1.24 Timber preservation.

Whilst decay of timber is widespread it is by no means inevitable and the long lasting timber constructions of man bear witness to the inherent durability of timber. Essentially fungi can decay timber when moisture levels increase above about 20% (Coggins, 1980). A variety of preservative methods have been devised for situations where it is impossible to prevent moisture levels from reaching this value. The methods in use can be categorised under one

of the following headings: non-pressure, diffusion and sap replacement; or pressure and vacuum impregnation (Kollman & Cote, 1968). All these processes involve impregnation of wood with a biocidal chemical. The chemicals which are applied in these methods include organic preservatives and inorganic water soluble preservatives, e.g. boron and fluoride compounds or compounds of copper-chrome-arsenic (CCA). The most widely used organic preservative is creosote but other compounds which are used as fungicides are pentachlorophenol and tributyltin oxide and derivatives (Becker, 1968) in suitable solvents.

Creosote, an oil from coal tar, was introduced for practical purposes in 1883 when Bethell's patent for the use of 'dead oil' of tar was taken out. Initially it was principally used for the preservation of railway sleepers and wooden ships (Ramsbottom, 1937). Creosote has long been regarded as a standard preservative for woodwork of low aesthetic value and is used for most distribution and telegraph poles in the UK; fence posts, garden sheds and hoardings are often routinely coated with creosote. Preservation of domestic building timber is rarely undertaken despite either the inevitability of decay if timber is wetted or the relative cheapness of preservation when compared with the total cost of replacement. Recent figures from the Forestry Commission Timber Utilisation Department suggest that it would only cost £120 to preserve all the timber in a new house.

1.1.25 Alternatives to chemical preservation.

A criticism of any chemical preservative method is that the chemicals used are necessarily biocidal and potentially dangerous to humans, other animals and plants but searches for less environmentally dangerous preservatives are ongoing

in many laboratories. An alternative to using potentially dangerous chemical preservatives would employ a biological organism which is a natural predator on the decay fungus only, i.e. a control agent which would ideally be target specific. These biological control methods have the potential to control pest organisms in an environmentally safe way because they exploit the fact that every organism is part of a food web, thus implying that the pest is preyed upon and used by another organism as a food source. Biological control has been defined as the suppression of a pest by means of the introduction of the predators, parasites and the diseases by which it is naturally attacked (Smith, 1948). Biological control has been widely used in many areas of horticulture but has been infrequently investigated for control of Basidiomycetes. Bruce (1983) investigated the effect of Trichoderma and other moulds on control of L. lepideus in creosoted distribution poles and A. Score (personal communication) found that T. harzianum stopped the growth of isolates of the dry rot fungus S. lacrymans.

1.2 The dry rot fungus.

1.2.1 Serpula lacrymans.

S. lacrymans (Wulfen: Fr.) Schroeter apud Cohn is perhaps the best known brown rot Basidiomycete and is certainly the most destructive wood decay fungus of buildings in temperate regions outwith the USA (Cartwright & Findlay, 1958). It is the causative organism of dry rot of timber and, together with 2 wet rot fungal organisms, C. puteana and F. vaillantii, is responsible for 95% of decay of building timbers in Britain (Coggins, 1980). The use of the term 'dry rot' has its origins in the times even before it was recognised that a fungus played any part in the decay (Coggins, 1977) and S. lacrymans has come to be exclusively associated with this term.

1.2.2 Nomenclature.

The name by which the organism is still known to many operatives working in timber preservation and architecture is Merulius lacrymans, which indicates that the nomenclature of S. lacrymans (S. lacrimans, M. lacrymans or M. lacrimans) is still in a confused state. Merulius as a generic name has been in use since 1720 but was sanctioned as a genus by Fries in 1821. However, in the same year, firstly, a distinction was established within the genus Merulius between the brown spored Serpula species and the white spored Merulius species and, secondly, Serpula was raised to generic status by Gray. There is variation in the literature over the spelling of the epithet lacrymans or lacrimans. In this thesis 'lacrymans' will be used because the original spelling by Wulfen (1781) was lacrymans. Fries (1821) corrected the spelling to 'lacrimans' because he was a Latin scholar but Article 73.1 of the International Code of

Botanical Nomenclature (1988) states 'the original spelling of a name or epithet is to be retained, except for the correction of typographic or orthographic errors' (Pegler, 1991).

1.2.3 Distribution.

S. lacrymans is common in most cool temperate regions of the world, with the notable exception of the USA where it is rare and the niche is occupied by Poria incrassata (Cartwright & Findlay, 1958). The restriction to temperate regions is usually explained by its sensitivity to heat since it cannot survive great temperature variation. S. lacrymans is usually considered to be a fungus which is found exclusively within timber buildings. Workers in Denmark (Bech-Andersen, 1985; Koch, 1991) and Australia (Thornton & Collett, 1983) have noted that the fungus usually grows in timber in close association with a calcium source e.g. mortar. However, between 1929 and 1952 it was reported growing in the wild on coniferous stumps and logs at 5 different locations in the Himalayas, at altitudes between 2400 - 3155m (Bagchee, 1954). Expert identification established that these specimens were S. lacrymans and not the closely related Serpula himantioides, which is usually stated to be the member of the genus associated with growth in the wild. Harmsen (1960) considered that S. lacrymans may be viewed as native to sub-tropical mountain forests at high elevation and indigenous to the California mountains in the USA. A report of S. lacrymans growing outwith buildings has also come from Czechslovakia (Soukup, 1979) where it was growing on the trunk bases of two living trees of Norway spruce in the Botanic Gardens near Prague.

1.2.4 Growth characteristics.

S. lacrymans grows between 3°C and 25°C with an optimum at 22°C (Langvad & Goksoyr, 1965). If it is exposed for 60 minutes to a temperature of 40°C it is killed (Falck, 1906) which is possibly a reason why S. lacrymans is absent from outdoor building timber, since sunlight can raise wood temperatures into the lethal range for S. lacrymans. It can grow 5 - 6mm per day (radial increase), at its optimum temperature, on an agar plate. However, its growth rate in buildings is difficult to define as it is affected by complexly interlinked factors, e.g. moisture levels within the wood, the relative humidity (RH) of the surrounding air, temperatures and degree of ventilation. Over a period of 50 days, when the air temperature varied between 3°C and 5°C, the radial increment of S. lacrymans was observed to be 2.25 mm per day, which would represent approximately 0.75 m in a year (Coggins, 1976).

Favourable conditions in a building for growth of mycelium are high RH, stagnation of air currents and low light levels. The mycelium stops growing if exposed to draughts which reduce the RH of the atmosphere. A RH of >93.5% was required for growth at a wood moisture content of >22% (Doi, Sato & Arima, 1982) but Brown, Fahim & Hutchinson (1968) showed that the fungus is capable of growing into wood blocks and causing weight loss at 90% RH but not at 82% RH. In 1885 Hartig noted that the fungus dies after only 10 minutes if exposed to air of medium RH.

The activities of S. lacrymans result in the lignin of infected wood becoming crumbly and dry to the touch but the term 'dry rot' does not indicate that growth of the fungus takes place in the absence of moisture. However, whereas the 2 wet rot fungi mentioned in section 1.2.1 only start to colonise wood at fibre saturation point (c. 26%), S.

lacrymans can colonise and begin to decay timber with a moisture content of 20%, although the optimum moisture content for its decay activities is in the range 30 - 40% (Coggins, 1980).

Prior to fungal colonisation wood has a pH in the range 4.5 - 5.5 but production of oxalic acid from the hyphal tips of S. lacrymans renders the wood in which it is growing highly acid; dry rotted wood being approximately pH 3.0 (Cartwright & Findlay, 1958). However, the drop in pH during fungal growth does not appear to retard it and growth continues vigorously until the nutrient source is exhausted (Cartwright & Findlay, 1958). There is evidence that the fungus may grossly affect the properties of plaster or mortar over which it is growing but this effect is not immediately apparent in normal dry rot infestations. The production of acidic droplets of pH 3 - 4 at the hyphal tips (Coggins, 1977; Brownlee & Jennings, 1981) play a part in plaster and mortar degeneration. Savory (1980) noted almost complete degradation of the calcium components of plaster blocks attached to pieces of wood which had been inoculated with S. lacrymans. No conclusive evidence has been presented which would indicate whether the damage to calcium containing materials is metabolically essential to create an optimum pH in the micro-environment for growth; or, whether the damage is incidental and concomitant with the normal metabolic function of oxalic acid excretion. Bech-Andersen (1985) noted that dry rot attacks in Copenhagen were never more than 1 m away from a source of calcium. He has argued that S. lacrymans is found close to sources of calcium in order to neutralise the very acid conditions generated as a result of the production of oxalic acid and, thereby, create an optimum pH in the environment for growth. In possible support of this is the observation of crystals of calcium

oxalate on certain hyphae and strands of S. lacrymans (Nuss, Jennings & Veltkamp, 1991).

1.2.5 Economic importance.

The economic importance of S. lacrymans is confined to its effects upon man's timber constructions. In the UK, before the second world war, it colonised oak and other native hardwoods which were then commonly used for building purposes. Declining stocks, and concomitantly increasing costs, of this type of timber have meant that coniferous timbers are used routinely now for building purposes so it is more usual nowadays to find an outbreak in softwood timber. Inside buildings it has catholic tastes, not only exploiting wood but also damp paper, cardboard and other cellulose containing materials. Another important aspect of its success as a pest of buildings is its ability to spread through non-nutrient materials, such as plaster and brickwork, which intervene between nutrient sources.

Today, S. lacrymans causes millions of pounds worth of damage annually worldwide. For example, in the UK the estimated cost of rectifying damage caused by S. lacrymans is at least £150 million per annum (Jennings & Bravery, 1991); in Norway it was calculated that the organism annually caused four times as much damage as fires (Coggins, 1980); and, on a smaller scale, in 1988 an outbreak in Dundee caused £15,000 worth of damage to a building whose construction costs had been £9,000 only eight years previously (Mr. F.G. Allan, personal communication).

1.2.6 Initial infection of building timber.

S. lacrymans enters a building by aerial dispersal of spores (basidiospores) from an infected site. They do not germinate on dry wood but only on timber which is

sufficiently wet. Spores are approximately 9.7 µm in length and 5.4 µm in breadth (Falck, 1912) with a thick brown wall which has a thin portion called the apiculus. Ultrastructural studies (Hegarty & Schmidt, 1988) on mature spores have indicated that approximately 40% contain a single nucleus in cytoplasm consisting of vacuoles, mitochondria and endoplasmic reticulum. The remaining spores generally lack a nucleus and consist of vacuoles filled with lipid-like material. During germination the germ tube grows out of the apiculus. The resulting primary mycelium is composed of monokaryotic hyphae with a diameter of 1 - 1.5 µm and no clamp connections. This mycelium can grow either on or within the timber and normally neither spore germination nor the resulting monokaryotic mycelium on timber is visible to the naked eye.

The first visible signs of the dry rot fungus are white patches of secondary mycelium which result from the confrontation of two monokaryotic mycelia. The change from monokaryotic to dikaryotic mycelium is morphologically characterised by an increase in hyphal diameter to approximately 6 µm) (Falck, 1912) and the formation of clamp connections.

A second mode of infection has implications for building remedial work; if fresh wood is placed against building material (wood, plaster or brickwork) which is infected with S. lacrymans it can become colonised with dikaryotic mycelium and a fresh 'flush' of mycelial growth is initiated.

The leading edge of the mycelium continually grows from the point of inoculation and simultaneously exploits the substrate. Once established inside a building the mycelium displays an adaptation which is possessed by no other Basidiomycete and which enables it to invade a potentially

xerophytic environment with a lower water content than the optimum required for growth. This is possible because the mycelium is known to routinely transport metabolic water forward in a translocation stream to the invading mycelial tip (Jennings, 1984), at high RH the metabolic water can appear as droplets on the surface mycelium, and it may be that unobserved exudation of metabolic water can modify a dry environment sufficiently for growth to occur. Interestingly, the derivation of the epithet '*lacrymans*' comes from the Latin 'lacrima', a tear; and *Serpula* is similarly derived from the Latin 'serpere', to creep or wind, which is descriptive of the mode of growth of the mycelium through the substrate.

1.2.7 *Morphogenesis of the mycelium*.

Morphogenesis can be defined as the development of an organism to a particular functional form, which implies differentiation of cells into types differing in their shape, size, structure and chemical composition (Turian, 1983). The morphological development of the surface mycelium of *S. lacrymans* from an inoculum has been described by Hornung & Jennings (1981) and was separated into 4 stages, within which 5 hyphal types, some present in more than one stage, were identified by light and electron microscopical studies. These stages of growth are Stage I which corresponds to the undifferentiated outgrowth from the inoculum; Stage II is when there is a white fur-like mycelium on the substrate; Stage III is when further outgrowth is inhibited and syrrotia (strands) start to become visible; and Stage IV is when the isolated syrrotia are macroscopically free from the rest of the colony. *S. lacrymans* produces 2 morphologically and physiologically different types of undifferentiated mycelium; one of which

produces strands and the other, tertiary mycelium, produces fruit bodies (Nuss, Jennings & Veltkamp, 1991). The strand forming type has been extensively investigated (Hornung & Jennings, 1981) since the component hyphal type is found in all morphological stages of the mycelium and is the precursor of the 4 differentiated types of hypha, viz main, tendril, fibre and vessel hyphae.

1.2.8 Stage I mycelium.

Hyphae in Stage I are undifferentiated and grow perpendicularly away from the substrate in bundles prior to collapsing. Contact of a bundle with a solid object leads to continued growth (Falck, 1912). Some of these hyphae produce crystals of calcium oxalate.

1.2.9 Stage II mycelium.

Stage II includes the growing margin of the colony and therefore represents 2 functional regions, i.e. the young actively growing absorbtive margin behind which is older mycelium where increasing vacuolation of cell contents occurs. The growing margin is composed of main hyphae and tendril hyphae whose joint functions are, firstly, exploitation of the environment by production of substrate degrading enzymes; and, secondly, forward growth by extension of the mycelial tip. Fungi whose sole nutrient source is wood need to be efficient at extracting, concentrating and conserving nitrogen (Levi & Cowling, 1969). The young mycelium of S. lacrymans is able to extract the nitrogen in wood and concentrate it (Watkinson, 1984a). As a conservation measure, nitrogenous fungal material is degraded in the older mycelium, prior to the return of the nitrogen in the translocation stream to the growing region of the mycelium for re-use (Watkinson, 1975). In support of

the autolysis of old mycelium is the difficulty of finding any hyphae on thoroughly decayed wood.

1.2.10 Stage III strand.

Stage III in the development of the mycelium represents a stage of differentiation which is associated with the appearance of strands. This is a morphological stage which is visually distinct from Stages I and II, is characteristic of many Basidiomycetes and is accompanied by changes in the pattern of enzyme proteins (Wong & Willets, 1973) and activities (Chet, Retig & Henis, 1972). The early development of strands was described by Butler (1957, 1958). They are a composite of 3 mycelial types and are initiated approximately 1 cm behind the colony margin in a process in which the wide main, empty looking vessel hyphae start to become ensheathed by thick walled, occluded, impermeable fibre hyphae. Both of these are bound together with narrower metabolically active tendril hyphae which results in adhesion between the 3 hyphal types (Hornung & Jennings, 1981) which is possibly aided by impermeable, extra-hyphal fibrillar material between them (Jennings & Watkinson, 1982). At this early stage in strand formation (Stage III) there is much Stage II mycelium between the syrrotia.

1.2.11 Strand initiation.

Strand material can be formed behind the mycelial margin when growth of the mycelium is inhibited. It can be initiated in the laboratory by growth over a staled or toxic medium; by growth from a food base through non-nutrient medium; or by contact of the hyphal margin with a fresh food base (Butler, 1958). Watkinson (1971a) found that strand formation in S. lacrymans is increased by a nitrate containing medium.

A two fold function is envisaged for strands. Firstly, the formation of impermeable strands from the permeable stage II mycelium is possibly a means of conserving nitrogen. In support of this, Watkinson (1975) noted leakage of nitrogenous material from autolysing stage II mycelium and found that this leakage was partly responsible for strand initiation. Autolysis of old mycelium may be concomitant with increased permeability of the hyphal wall and loss of essential nitrogenous material from the colony. Secondly, since strands function in translocation of soluble metabolites (Watkinson, 1971b) they might aid in recycling nitrogen by acting as a connection between autolysing mycelium on an exhausted substrate and mycelium on a secondary substrate which is undergoing colonisation (Watkinson, 1975).

1.2.12 Stage IV strand.

During the ageing process the production of slime proceeds at the expense of tendril hyphae which continue to excrete calcium oxalate crystals in the strand periphery. An increasing number of hyphae collapse within the strand and form longitudinal, hollow channels of approximately 25 µm in diameter which are embedded in matrix material (Jennings & Watkinson, 1982). In the field situation thick walled strand material is formed which acts as a link to conduct food and water to the colonising mycelium from the regions of substrate utilisation. This enables the mycelial form of the fungus to traverse barriers, such as bricks and mortar, so that new timber may be colonised whilst the established mycelium is still within an exploitable timber substrate. The adaptive advantage of strands may be that they are the most efficient means of supporting mycelial advance from a

distant food base (Watkinson, 1979). A mature strand may be up to 1 cm in diameter and may reach a length of 4 m.

1.2.13 Fruit body formation.

Fruit body (basidiocarp, sporophore) formation rarely occurs spontaneously in the laboratory but in the field it typically occurs when food material is exhausted. They can form all the year round but under field conditions the majority of fruit bodies are produced in spring and autumn. Fruit bodies usually form on a wood/wall joint and do not have any distinct shape and size. No studies have produced definitive information on the requirement of light for fructification since the precise influence of light or darkness is difficult to monitor under field conditions (Hegarty, 1991). Young fruit bodies are composed of intercellular spaces surrounding strand-like structures. These differ from strands in that they are composed of only one type of hypha which run parallel to each other, have many clamp connections and are bound together with mucilage (felted hyphae) (Nuss, Jennings & Veltkamp, 1991). At a later stage the hymenium (fruit bearing layer) starts to develop. It is composed of vertical hyphae which eventually produce basidia directly exposed to the atmosphere and bearing 4 basidiospores. Falck (1913) estimated that at maturity a fruit body 100 cm^2 can produce 50 million rusty-red spores in 10 minutes.

1.2.14 Historical records.

Given the economic importance of S. lacrymans it is hardly surprising that a great deal has been documented historically about this fungus. It has been suggested that the dry rot of timber described in the Bible as 'leprosy of house' (Leviticus, Chapter 14) was caused by this fungus but

this is unlikely since the average temperature in Palestine is probably too high for the growth of S. lacrymans (Cartwright & Findlay, 1958). It can be assumed that the activities of S. lacrymans were behind a comment in one of Francis Bacon's essays (1612), viz 'it is a reverend thing to see an ancient castle or building not in decay'.

Britton described this fungus in 1875 as 'one of the most formidable of the tribe of fungi'; a very apt comment to make if it were based on observations of the havoc wrought in previous years by this fungus upon the ships of the Royal Navy. Two of the best accounts of S. lacrymans and its effects between 1600 and 1863 upon naval ship timbers were written by Albion (1926) and Ramsbottom (1937). Both accounts emphasise that especially during the period of the Napoleonic wars from 1803 - 1815 ships were built hastily and mainly of 'green' (moist) unseasoned oak timber. As a result some timbers started to decay even before the ships were launched and the periods of idleness between intermittent wars saw ships rotting and sinking at their anchors because of the ravages of S. lacrymans.

Most notable was the 'Queen Charlotte' which was launched in 1810 at a cost of £88,500 but which needed to be repaired even before going to sea. By 1816 the repair bill had amounted to more than her original cost and by 1859 the total cost of the repairs was £287,837. Another description from Ramsbottom (1937) which further illustrates the activities of this fungus is 'about 1789 there was a ship in so bad a state that the deck sunk with a man's weight and the orange and brown coloured fungi were hanging in the shape of inverted cones from deck to deck'. After 1863 ships started to be constructed of iron and the economic effects of S. lacrymans upon the navy became neglible.

As a result of the effects of S. lacrymans on ships the Royal Society of Arts offered prizes for a cure for dry rot. Most solutions related to land based timber usage, e.g. Batson (1794) and Britton (1875), and implies that the organism was well known as a pest of domestic timber even though there is no generally available historical account of outbreaks of S. lacrymans in domestic timbers from 1863 until after the first world war. It seems probable that the first outbreaks of dry rot in domestic timbers were caused by use of unseasoned timber and bad ventilation. A few investigators recognised the link between these conditions and dry rot but their advice to the effect that working practices should change was not heeded. S. lacrymans outbreaks became increasingly common during, and after, the first world war due to neglect of upkeep, novel methods of building construction and use of unseasoned, especially coniferous, timber.

Attacks escalated during, and immediately after, the second world war because many buildings were left empty and exposed to wet conditions after serious air raid damage (Cartwright & Findlay, 1958). So long as the woodwork in a building remains dry it will remain sound but timber decay is inevitable as soon as moisture gains access to the timber. This connection between environmental conditions and fungal activity is now well recognised but, in spite of this, continued outbreaks of S. lacrymans up to the present time indicate that lessons have not been fully learnt (Coggins, 1980). New outbreaks nowadays are partly due to bad design, use of unseasoned coniferous timbers and bad maintenance; and partly due to bad renovation following a previous outbreak of S. lacrymans in damp, badly ventilated buildings.

1.2.15 History of morphological studies.

A precise macroscopical description of the development and morphology of S. lacrymans was given by Accum in 1827 (Buhler, 1845). From then until 1912 many contributions were made, particularly by German scientists, to the morphological knowledge of S. lacrymans. These included 2 notable monographs; the excellent microscopist Hartig (1885) published what is acknowleged to be the first fundamental scientific monograph on S. lacrymans and this was followed by that of Falck (1912) on the physiology, morphology and germination of the fungus. Many morphological studies followed, including Butler's investigations on strands (1957, 1958). Recent electron microscopical work has elucidated the hyphal makeup of mycelium and strand (Hornung & Jennings, 1981) and fruit body (Nuss, Jennings & Veltkamp, 1991).

1.2.16 Recent physiological studies.

The physiology of the organism has been extensively studied. Recent investigations have been made into strain characteristics (Abou-Heilah & Hutchinson, 1978; Wazny & Thornton, 1991) and the wood rotting abilities of strains (Diller & Koch, 1959; Thornton, 1985); comparisons between dikaryons and monokaryons (Elliott et al., 1979) and between growth rates and decay ability (Bravery & Grant, 1985; Thornton & Wazny, 1986; Cymorek & Hegarty, 1986; Thornton, 1989); translocation (Watkinson, 1971b; Brownlee & Jennings, 1982); water relations (Eamus & Jennings, 1986); production and location of proteinases (Venables & Watkinson, 1989); and utilisation of soil components (Doi & Togashi, 1990).

1.2.17 Studies on prevention and cure.

Dry rot should never develop in a new building. Therefore design and maintenance of a new building should aim at the exclusion of all external moisture since S. lacrymans can develop in any part where persistent leakage of water occurs and good building practices are of prime importance in prevention of dry rot of wood. This was recognised as long ago as 1794 when Batson recommended removal of earth from the base of damp walls in which timber had decayed. Branley (1803) advised using well seasoned timber and advocated adequate drainage of the land round a building. Britton (1875) provided a summary of advice which is as applicable today as it was then, viz 'season and ventilate'. The latter is especially important in areas such as cellars which are associated with dampness.

S. lacrymans is very sensitive to antiseptics and its growth is readily checked by comparatively low concentrations of most wood preservatives. Infected masonry can be treated by application of water- or solvent-soluble solutions of chemical preservatives e.g. mercuric chloride, zinc oxychloride, sodium pentachlorophenate or sodium orthophenylphenate (Cartwright & Findlay, 1958). Timber, especially that which is to be used for ground floor joists and wall plates, should ideally be pressure impregnated with preservatives. Creosote and tar oils have been used routinely for these purposes if the timber subsequently does not have to be painted and if it is not going to be exposed to foodstuffs; in these instances either water- or solvent-soluble preservatives are used, such as those previously listed for the treatment of infected masonry.

There have been many papers published relating to the use of chemicals in the prevention of wood decay by S. lacrymans. These range from Batson (1794), who suggested

covering soil with anchor smiths' ashes, to more recent work on the preventative effects of creosote oil (Doi, 1989); zinc oxychloride (Coggins & Jennings, 1975); copper-chrome-arsenate (CCA) and sodium pentachlorophenate (Wazny & Thornton, 1986); 4-chlorophenyl-3-iodopropagylformal and tolclofosmethyl (Takahashi & Nishimoto, 1985); tributyltinoxide, 4-bromo-2,5-dichlorophenol and chloronaphthalene (Doi, 1989);.

1.2.18 Novel aspects of control.

Little work has been initiated into biological control of S. lacrymans even though Bruce (1983) found that the deuteromycete fungus Trichoderma had the potential to be investigated as a biological control agent of the organism. Elliott & Watkinson (1989) investigated the effect of an amino acid analogue α-amino-isobutyric acid (AIB) on S. lacrymans. AIB was found to inhibit hyphal tip extension growth and to interfere with normal morphogenesis by increasing the frequency of mycelial branching; and it also had a preservative effect when impregnated into wood blocks. Later studies (Watkinson, 1984b) found that AIB was neither metabolised nor incorporated into protein. These studies indicate that AIB has potential for either wood preservation or control of the established organism.

1.2.19 Treatment of infected buildings.

Primary measures involve the location and elimination of sources of moisture and the promotion of rapid drying of the structure by increase of ventilation and heating. Secondary control measures for S. lacrymans involve the removal of all rotted wood to 0.45 m beyond the last indication of infection and the use of preservative treated replacement timbers (Bravery et al., 1987). The secondary measures need

not be so drastic if misdiagnosis has occurred, but operatives must always operate on the side of caution so there must be many instances where too much remedial action is taken to the economic disadvantage of their clients.

Since S. lacrymans is sensitive to high temperatures a method which is in the early stages of industrial assessment has been devised to heat treat infected buildings (Koch, 1991). After sources of moisture have been eliminated the affected parts of the building are heated at 40^{0}C for 24 hours with the aim of killing mycelium at the surface and within timber and masonry.

1.2.20 Detection of S. lacrymans.

Observation of either a fruit body, mycelium or decayed wood may be the first indication of an outbreak of S. lacrymans, by which time the structural integrity of the timber has been seriously compromised. Subsequent visual surveys to determine the extent of the outbreak often involve destructive exposure work behind timber constructions. Visual inspections are relatively unsophisticated and are aided by the use of torches, hand mirrors, endoscopes and fibre optics to inspect comparatively inaccessible voids. Subsequent microbiological examination of cores and mycelial material would confirm the outbreak. However, such examinations can be time consuming and are rarely done by commercial operatives since by these stages outbreaks are well established.

Non-destructive detection of decay is desirable on economic grounds and non-destructive testing (NDT) methods are being evaluated for their usefulness in the detection of the fungus during the earlier stages of its development before gross structural damage has been done within a building. NDT methods include the use of specially trained

sniffer dogs (Hutton & Rostron, Guildford, Surrey; Madsen & Adelhoej, 1989); and ultra-sonics, Eddy current, thermal emission, X-ray analysis and collimated photon scattering (CPS) (Madsen & Adelhoej, 1989): to date, CPS and sniffer dogs have promise for NDT of wood for S. lacrymans (Koch, 1991).

1.2.21 Early detection of S. lacrymans.

It would be even more desirable on economic grounds to be able to detect S. lacrymans prior to any structural damage. At an early stage of infection the smaller mass of infective organism could be treated more efficiently with the use of less drastic remedial treatment methods. This is to be desired not only on the grounds of the expense of remedial treatment but also on safety grounds. However, at an early stage of decay the fungal organism is present only in very small amounts, is not itself visible and does not produce gross visual changes in wood. Detection of the infective organism at an early stage of morphological development poses considerable challenges to the traditional detection methods currently used.

In recent years the development of molecular analyses based on biochemical and immunological techniques have facilitated the detection, identification and assay of an enormous range of biological macromolecules and whole organisms. These methods can often operate successfully even when the analyte of interest is present in very small amounts within a sample.

1.3 Molecular analysis.

1.3.1 Protein electrophoresis.

Protein analyses are routinely carried out for the detection and identification of micro-organisms and for the study of specific proteins. Two linked techniques are used for this purpose; polyacrylamide gel electrophoresis (PAGE) and silver staining; PAGE electrophoretically separates the constituent native proteins in a sample and silver staining visualises these proteins. When used in combination with each other these techniques can detect levels of protein in the nanogram range (Blum, Beier & Gross, 1987). The production of electrophoretic patterns of soluble proteins extracted from fungal mycelia has been widely used in the identification and classification of fungal isolates (Clare, 1963; Bent, 1967; and Hansen et al., 1986).

The technique of separation of native proteins by PAGE was introduced independently by Slater (1965, 1969) and Margolis & Kenrick (1967, 1968). Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a refinement of PAGE. The sample whose protein content is to be analysed is heated prior to electrophoresis with a buffer (boiling mix) containing mercaptoethanol and sodium dodecylsulphate (SDS). The SDS solubilises the proteins whilst the mercaptoethanol reduces disulphide linkages between protein subunits and releases them as monomers. The addition of SDS to the acrylamide mixture in the resolving gel creates a denaturing gel which results in a subsequent separation by electrophoresis that is based on the molecular weight of the monomers and not on the shape or charge of the native proteins. Since the first investigations which used the continuous buffer systems of Shapiro, Vinuela & Maizel (1967) and Weber & Osborn (1969) the technique of SDS-PAGE

has been continuously developed to provide better separation of protein subunits within a specified molecular weight range. The Laemmli (1970) system represents one of the most common buffer systems used today. This technique has been used in mycology to identify aggressive and non-aggressive strains of C. ulmi (Jeng & Hubbes, 1983) and to identify different species of Phytophthora (Hansen et al., 1986).

1.3.2 Immunological detection techniques.

These feature a wide range of simple techniques based upon, and associated with, the development of immunological reagents. There can be two different aims in the immunodetection of macromolecules. The first is the need to quantitatively assess an immunoreagent (either antibody or antigen) and the second is the need to investigate the molecular nature, purity and molecular size of the antigen itself. Systems which quantitatively measure immunoreagents are generally called immunoassays whilst those which investigate the nature of the antigen are techniques which fall into the area of immunoanalysis.

These immunological techniques are based upon antibodies which are glycoproteins produced by the immune system of vertebrates and evolved primarily as a defence against infection. They are produced when a molecule, an antigen, which is not intrinsic to the species enters the animal's body and stimulates B-lymphocytes to produce antibodies. Since most complex molecules with molecular weights >5000 Daltons can be antigenic it is possible to immunise animals with an antigen of interest and produce serum containing antibodies (antiserum) against this antigen. The antibody has binding sites which specifically recognise and bind to the antigenic molecule which induced its production and binding occurs even when the antigen is present in very low

amounts. This function is exploited by immunotechnologists in their use of antibodies as molecular probes. Antibody based techniques are suitable for the detection of macromolecules and are extremely sensitive. Under optimal conditions antibody based detection systems can detect fewer than 1000 molecules of antigen (Roitt, Brostoff & Male, 1985) which means that immunodetection systems are, for example, more sensitive at revealing the presence of specific proteins than SDS-PAGE/silver staining techniques. Such methods lend themselves to the detection of the very low levels of fungal biomass which are present in the early stages of fungal decay.

1.3.3 Immunoassays.

Immunoassay is widely used in medical diagnosis of fungal related disease (Seeliger, 1982). Such techniques have also been applied to the determination of plant pathogenic fungi (Walcz et al., 1985) and the estimation of fungal biomass in leaf litter decomposition studies (Frankland et al., 1981). Immunoassay systems for the early detection of Basidiomycetes have been developed for the brown rot fungi Lentinus lepideus (Glancy et al., 1989) and P. placenta (Goodell & Jellison, 1986) whilst the detection of white rot fungi such as Coriolus versicolor has also been described (Palfreyman et al., 1987).

One area of immunoassay involves the use of a solid phase together with enzyme-coupled reagents. These assays exploit the extremely high catalytic power of enzymes for the amplification of the final signal prior to detection of the molecules of interest. Sensitivities can be in the range 0.1 - 10 ng of the antigen depending on the system, although autoradiographic and fluorographic methods can be 10 - 100 times more sensitive even than this (Stott, 1989). Enzyme

immunoassays have many of the properties of an ideal immunoassay which should be versatile, robust, simple to perform; and should use stable reagents economically and achieve a simple separation of bound and free reagents (Catty & Raykundalia, 1989). Various modifications have been developed using enzyme labelled antibodies and one technique is the enzyme-linked immunosorbent assay (ELISA), so called because one immunoreagent is absorbed to a solid phase prior to incubation with the immunoreagent to be quantified and the subsequent use of the enzyme labelled antibody.

A variety of solid phase surfaces have been used since the earliest tests which relied on plastic tubes. These have largely been replaced by plastic microtitre plates and recent modifications include solid phase reactions on micro-pegs, dip sticks, beads and protein binding membranes such as nitrocellulose or polyvinyl difluoride. One type of ELISA is the enzyme labelled anti-globulin assay (indirect ELISA) which involves a solid phase bound antigen with which the antibody of interest is incubated prior to detection by labelled anti-globulin. When this indirect ELISA is carried out with spots (dots) of the antigen bound to a protein binding membrane it is usually called a dot blot (or dot ELISA, dot immunobinding or immunodot) assay.

The dot blot assay was developed by Towbin & Gordon (1984) and, since it can provide basic information on the antigen:antibody reaction and can detect <10 ng/ml of antibody in serum (Catty, 1988), it has potential for assessing the titre of the desired antibody in serum since it provides a relatively simple and quick screening procedure.

1.3.4 Immunoanalysis.

A major type of immunoanalysis is western blotting, immunoblotting, which visualises antigenic proteins after the constituent proteins in a sample have been separated by SDS-PAGE and electrophoretically transferred to a protein binding membrane. This method of immunodetection allows constituent proteins which contribute to the molecular nature of the antigen to be investigated and determines the difference between antigenic epitope not only by visual interpretation but also by a more accurate molecular weight determination.

The technique was christened western blotting by Burnette (1981) but was initiated in 1979 when Towbin, Staehelin & Gordon transferred proteins electrophoretically from polyacrylamide gels onto nitrocellulose membranes and probed the antigenic proteins in a similar manner to that used in the indirect ELISA. Since then there have been many developments in the immunoblotting technique but the essential basics remain the same. It has been used in a great variety of studies including, in the field of mycology, the identification of plant pathogens such as Phytophthora species (Hansen et al., 1986) and the identification of the Aspergillus fumigatus isolates responsible for invasive aspergillosis in humans (Burnie et al., 1989).

Western blotting is a highly sensitive and discriminatory technique which has potential to allow comparisons to be made not only between different isolates of S. lacrymans, which may differ little from each other in terms of their general protein composition, but also to investigate antigenic differences between S. lacrymans and other species of fungi.

1.4 Description and aims of the project.

This project was initiated to apply methods of molecular analysis to the dry rot fungal organism, S. lacrymans. There is a need to develop a routine method of identifying incipient decay by S. lacrymans. The ideal method would meet several criteria, viz accuracy in identification; faster than current identification methods which use microbiological techniques; and sensitive enough to detect the decay organism at a stage that permits remedial treatment before large scale damage and strength losses have occurred. Molecular methods have the potential to fulfill these criteria. Identification is dependent upon knowledge of the molecular nature of an organism so two closely related techniques, biochemical and immunological, were evaluated for their ability to contribute to an understanding of S. lacrymans in laboratory and field material.

The specific aims of the project were

1) to investigate the protein, glycoprotein and antigenic nature of isolated S. lacrymans and to compare this organism with other wood decay Basidiomycetes;
2) to investigate the effects of substrate and other growth conditions upon the protein and antigenic nature of isolates of S. lacrymans;
3) to develop molecular probes for S. lacrymans which could further the understanding of S. lacrymans and;
4) to initiate a comparison at the molecular level of laboratory and field isolates of S. lacrymans.

CHAPTER 2. MATERIALS AND METHODS.

SOURCE OF EQUIPMENT AND MATERIALS.

Suppliers of equipment and materials are listed in Appendix A (A1) and the reagents required for techniques mentioned in this chapter are also listed in Appendix A (A2 - 32). In this chapter each part of Appendix A is mentioned when it is first relevant.

1. FUNGAL ISOLATES.

The 19 strains of S. lacrymans, 22 additional Basidiomycetes and 6 non-Basidiomycetes which were used in this study are listed in Tables 1 and 2. Sources of the fungi are shown in Table 3; each collection is represented by the alphabetic prefix in the strain number of each species. DIT strains were cultured from basidiocarp material collected locally.

2. CULTURE OF ORGANISMS.

2.1 Temperature for growth.

All S. lacrymans isolates, C. puteana, Paxillus panuoides, Daedalea quercina, and Serpula tignicola were cultured at 22°C in a cooled incubator (Gallenkamp cooled incubator). All other fungal isolates were cultured at 25°C.

2.2 Routine maintenance of fungal organisms.

Cultures of fungal isolates from the culture collections were inoculated onto 5% (w/v) malt extract (ME) /2% (w/v) purified agar (5% ME/2% agar) (Appendix A, A2)

Table 1: Strain number, original date of isolation and place of isolation of the Serpula lacrymans isolates used in the study.

Strain number	Isolation date	Source
FPRL 12C	-	FPRL, U.K.
CMI 152233	-	CMI, U.K.
BF-01	1965	France.
BF-03A	1936	Verdingen, Germany.
BF-07B	1967	U.K.
BF-015B	-	German Democratic Republic.
BF-017B	1937	France.
BF-018A	1939	Germany.
BF-023	1984	German Democratic Republic.
BF-025	1984	German Democratic Republic.
BF-044	1946	U.S.A.
BF-046	-	CSIRO, Australia.
BF-049	1981	Australia.
BF-050	1981	Australia.
BF-072	1930's	Germany.
DIT-101	1989	Dundee, Scotland.
DIT-102	1989	Dundee, Scotland.
MAD 90876-R	-	-
BAM 315	-	-

Table 2: Fungal organisms used in the study.

BROWN ROT BASIDIOMYCETES.

Amyloporia xantha (Fr) Bondartsev & Singer ex Singer	FPRL 62F
Coniophora puteana (Schumacher ex Fr) Karsten	FPRL 11E
Daedalea quercina L ex Fr	FPRL 38
Fibroporia vaillantii (DC ex Fr) Parm	FPRL 14G
Gloephyllum sepiarium (Wulf ex Fr) Karsten	FPRL 10D
Gloeophyllum trabeum (Pers ex Fr) Murrill	BAM(EDW) 109
Lentinus lepideus (Fr ex Fr) Fr	FPRL 7H
Merulius tremellosus (Schrader) Fr	FPRL 13
Paxillus panuoides (Fr ex Fr) Fr	FPRL 8B
Peniophora gigantea (Fr ex Fr) Massee	FPRL 175B
Poria incrassata (Berk & Curtis) Burt	FPRL 71
Poria placenta (Fr) Cooke sensu J. Eriksson	FPRL 280
Serpula himantioides (Fr ex Fr) Karsten	FPRL 233C
Serpula lacrymans (Wulf:Fr.) Schroeter apud Cohn	see Table 3
Serpula pinastri (Fr) Cooke	FPRL 141B
Serpula tignicola (Harm) Christians	CBS 311.54

WHITE ROT BASIDIOMYCETES.

Coriolus versicolor (L ex Fr) Quelet	FPRL 28A
Daldinia concentrica (Bolt ex Fr) Ces & de Not	FPRL 26E
Heterobasidion annosum (Fr) Bref	FPRL 41E
Pleurotus ostreatus (Jacq ex Fr) Kummer	FPRL 40A
Schizophyllum commune Fr	FPRL 9
Stereum sanguinolentum (Alb & Schw ex Fr) Fr	FPRL 27D

NON-BASIDIOMYCETES.

Cladosporium resinae	BM-13385
Paeciliomyces variotti	pole isolate
Trichoderma harzianum Rifai	IMI 206040
Trichoderma longibrachiatum Rifai	IMI 53608
Trichoderma polysporum (Linx ex Persoon) Rifai	IMI 206039
Trichoderma saturnisporum Hammill	IMI 14685

Table 3: Source of fungal isolates used in the study.

Prefix to isolate	Culture source
BAM	Bundesanstalt fur Materialprufung, Berlin-Dahlem, Germany.
BF	Dr. B. Hegarty, Rohm & Haas France S.A., Valbonne, France.
BM	Professor T. Nilsson, Ultuna, Sweden.
CBS	Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
CMI, IMI	CAB International Mycological Institute, Kew, U.K.
CSIRO	Commonwealth Scientific and Industrial Research Organisations, Melbourne, Australia.
DIT	Dundee Institute of Technology, Dundee, U.K.
FPRL	Department of the Environment Building Research Establishment, Garston, U.K.
MAD	Forest Products Laboratory, Madison, Wisconsin, U.S.A.

slopes and incubated at the appropriate temperature until the agar surface was covered with mycelium. Stock slopes were stored in the dark at 4°C and subcultured every 6 months.

All fungi were cultured at the appropriate temperature on 5% ME/2% agar plates (Appendix A, A2). With the exception of S. lacrymans all inoculation cores were placed so that the fungus was in contact with the agar surface.

2.3 Growth of mycelium for analysis.

8 different methods of culture were used to prepare mycelium for analysis.

2.3.1 Agar grown mycelium.

Agar grown mycelium was prepared by inoculation onto 5% ME/2% agar plates, cultured at the appropriate temperature until the plate was 75% covered. Subsequently, either 10 mm diameter cores were removed from the growing edge and used for inoculation purposes or the inoculation core was discarded and the remaining mycelium was scraped from the agar surface and stored at -20°C prior to freeze drying and analysis as agar grown mycelium.

2.3.2 Standard mycelium.

Standard mycelium was prepared from all the organisms in the study. A core of the appropriate fungus was inoculated into 5% (w/v) malt extract broth (MEB) (Appendix A, A3) so that the mycelium was uppermost at the surface of the broth. The MEB plates were incubated in static culture until approximately 75% of the broth surface was covered with mycelium (approximately 10 days

for S. lacrymans FPRL 12C). Harvesting was by removal of the mycelial mats from the broth, excision of the original inoculation core, shaking with 500 ml of ultra-pure (u-p) water and partial drying by vacuum filtration. The mycelium was washed with additional u-p water (500 ml) until the filtrate ran clear. Washed mycelium was collected in round bottomed flasks and stored at -20°C prior to freeze drying.

2.3.3 Young mycelium.

Young mycelium was prepared from agar grown fungal isolates which 75% covered a plate. The peripheral 5 mm of such cultures were harvested by carefully scraping the mycelium from the agar surface. Only S. lacrymans, C. puteana, P. incrassata, P. panuoides, F. vaillantii and C. versicolor could be harvested in this manner. No washing stages preceded the storage of this harvested mycelium at -20°C prior to freeze drying.

2.3.4 Aged mycelium.

Aged mycelium was prepared from agar grown fungal isolates which 75% covered a plate. The inoculation core was discarded and the 5 mm of mycelium round the inoculation area was carefully scraped off the agar. Aged mycelium was prepared only from S. lacrymans, C. puteana, P. incrassata and P. panuoides. The harvested mycelium was stored immediately at -20°C prior to freeze drying.

2.3.5 Variation in cultural parameters.

To investigate the effect of subculture, age, uplift period and washing on mycelium 3 consecutive subcultures of agar grown mycelium of S. lacrymans FPRL 12C were

made over the course of a two week period and used at 0 weeks, 2 and 6 months. Storage of the subcultures for use at 2 and 6 months was at 4°C. Subcultures were used as detailed for preparation of agar grown mycelium and standard mycelium but the time of harvest was altered. Inoculations from the 0 week subculture were harvested at 3, 5, 7 and 10 days (triplicate uplifts). At 2 and 6 months a further subculture onto 5% ME/2% agar preceded preparation of standard and agar grown mycelium which was harvested 10 days after inoculation (triplicate uplifts).

2.3.6 Mycelium in low nitrogen medium.

Nutrient solutions containing different concentrations of total nitrogen (TN) were prepared from medium composed solely of Sabouraud's liquid medium substitute (Appendix A, A4) or Sabouraud's liquid medium substitute and 24.8% (w/v) mycological peptone (Appendix A, A5). The final concentration of TN in each growth medium was equivalent to the TN content of 5% MEB (2.36%); the maximum TN in wood (0.127%); the minimum amount of TN in wood (0.03%); and 0%. Cores of S. lacrymans FPRL 12C were inoculated into these media and 5% MEB, grown and harvested in the manner previously described for standard mycelium.

2.3.7 Mycelium exposed to 40°C.

Agar plates which were 75% covered with S. lacrymans FPRL 12C were incubated at 40°C for 0, 1, 4, 6 and 24 h. At the end of the incubation period a core was cut from the growing edge and sub-cultured to test for viability. Young and aged mycelium was harvested from the remainder of the plate and stored at -20°C prior to freeze drying.

S. himantioides, optimum growth temperature 34°C (Schmidt, personal communication) was similarly cultured and harvested as a control organism.

2.3.8 Mycelium exposed to other fungal organisms.
Agar plates were inoculated near one edge with S. lacrymans FPRL 12C and cultured until the plate was 50% covered (approximately 7 days). Cores of Trichoderma longibrachiatum, T. saturnisporum or T. harzianum were inoculated at the opposite edge of the plate. These plates, and plates of S. lacrymans only, were cultured at 22°C. Young and aged mycelium of S. lacrymans FPRL 12C was harvested from these plates after 0, 1 and 2 days, pre-contact of the 2 organisms; and, after contact of the 2 organisms, at 3, 4 and 7 days.

2.4 Infection of wood with S. lacrymans FPRL 12C.

2.4.1 Preparation of wood.

Sapwood from Scots pine (Pinus sylvestris L.) and lime (Tilia vulgaris Hayne) was cut into 1 cm^3 blocks and sanded to remove loose pieces. These blocks were numbered, dried at 103°C for 3 h until they achieved constant weight and stored in a desiccator prior to recording of their dry weights (original dry weight). They were put into a desiccator and sterilised with ethylene oxide gas (10 ml liquid ethylene oxide/l of desiccator volume) for 24 h (Smith, 1965). A small volume of sterile water was also included in the desiccator to allow the blocks to moisten prior to subsequent fungal colonisation. The desiccator was vented in a sealed isolation hood for 48 h after sterilisation to remove all traces of ethylene oxide

from the blocks. This was important since it is known that residual ethylene oxide can have a deleterious effect on fungal growth (Dr. A.F. Bravery, Building Research Establishment; personal communication).

2.4.2 Infection of blocks.

Cultures of S. lacrymans FPRL 12C were grown on 5% ME/2% agar in 500 ml vented, screw topped glass jars until a mycelial mat covered the surface (approximately 2 weeks). Sterile plastic mesh was placed on top of the mycelial mat and 10 sterile wood blocks, either pine or lime, were added to the jar. The mesh prevented waterlogging of the blocks due to contact with agar. To reduce drying of the agar and to maintain adequate ventilation the jars were placed in a sealed, moistened tank inside a cooled incubator and gaseous exchange was maintained with an aquarium pump. Control blocks were placed on plastic mesh on sterile agar and exposed to the same conditions as test blocks.

2.4.3 Uplift of blocks.

The aim was to produce wood blocks at different stages of fungal colonisation and incipient decay; therefore blocks were harvested after 1, 2, 3, 5, 7, 9, 11, 14 and 16 weeks. These uplifts gave blocks with weight losses of from 0 - 61.99% (lime) and 0 - 42.5% (pine). After the appropriate incubation periods blocks were scraped free of surface mycelium, reweighed (uplift weight) and stored at -20°C prior to freeze drying. Blocks were stored in a desiccator after freeze drying, prior to reweighing (final dry weight) and storage of the blocks at -180°C until required for analysis.

2.4.4 Assessment of infection.

Assessment of infection of the blocks by S. lacrymans was investigated in 2 different ways.

2.4.4.1 Microscopical assessment.

Microscopical investigation involved differential staining (Appendix A, A6) of 10 µm tangential longitudinal sections (TLS) with safranin and picro-aniline blue stain (Cartwright, 1929) to confirm the presence of fungal mycelium (blue) in lignified material (pink).

2.4.4.2 Weight loss.

Infection of the blocks by S. lacrymans was confirmed by % weight loss measurements which were calculated after the % moisture content of the block had been established in order to ensure that decay by S. lacrymans had occurred at appropriate moisture levels. These measurements were calculated for each block using the standard formulae (Wilkinson, 1979), viz

$$\% \text{ moisture content} = \frac{\text{uplift weight - final dry weight}}{\text{final dry weight}} \times \frac{100}{1}$$

$$\% \text{ weight loss} = \frac{\text{original dry weight - final dry weight}}{\text{original dry weight}} \times \frac{100}{1}$$

3. FIELD STUDIES.

3.1 Sites of collection of field samples.

Samples of *S. lacrymans* were collected from 10 different sites in Scotland and Germany (Table 4).

3.2 Field notes.

The visual appearance of each sample was recorded as either 'fresh' or 'desiccated'. The former indicated that the sample was moist (mycelium or basidiocarp) and was harvested from an actively growing flush (mycelium) or possessed spores (basidiocarp). Strand material could not easily be assessed in such a manner but the term 'fresh' was applied to material from a site at which fresh mycelium was collected. 'Desiccated' indicated that the material looked opaque, dusty and old.

3.3 Sample collection.

Fungal samples and infected wood samples were collected and prepared for analysis. All samples were processed immediately after arrival in the laboratory by storage at -20°C prior to freeze drying.

3.3.1 Fungal samples.

Fungal field samples were personally collected from active outbreaks at sites A - C, whereas samples from sites D - G were supplied by Dr. B. Hegarty (Rohm & Haas France S.A., Valbonne, France) and were in a desiccated state after having been kept at ambient temperature for varying periods of time prior to processing. All fungal material was harvested as a complete uplift (composite samples) of the morphological form with the exception of only the 0.5 cm wide growing margin (young mycelium)

Table 4: Collection sites of field samples of Serpula lacrymans.

Site	Location	Building type
A	Dundee, Scotland	Private house
B	Brechin, Scotland	Church
C	Linlithgow, Scotland	Castle
D	Ebersbach, Germany	Church
E	Aulendorf, Germany	Castle
F	Erzingen, Germany	Town Hall
G	Engen, Germany	Church tower
H	Dundee, Scotland	Club house
I	Dundee, Scotland	Shop
J	Dundee, Scotland	Tenement house

from specific mycelial flushes (sites A - C); a mycelial flush of radius 46 cm, from site A, from which the young mycelium was harvested and successive 0.5 cm wide samples were taken at 2.0 cm intervals; and a 20 cm diameter basidiocarp from site D which was sampled 9 times at successive distances from the initial sample at the margin. The total number of samples collected and analysed were 49 mycelial samples, 29 basidiocarp samples and 9 strand samples.

3.3.2 Wood samples.

Samples of putatively infected wood were collected personally from sites of active outbreak in Scotland (sites A - C, H and J) or were supplied in a desiccated state from either Germany (sites D and E) or Dundee (site I). The latter samples were supplied by Mr. F.G. Allan (Ritchie, Dagan & Allan, Chartered Architects, Dundee) and consisted of 6 samples which had been drilled at various distances from an infection point within a softwood beam, of cross-section 20 cm x 25 cm, which had been diagnosed infected some 6 years previously. 20 infected wood samples were analysed.

4. FURTHER SAMPLE PROCESSING AND STORAGE.

All fungal material for electrophoresis was freeze dried using a vacuum freeze drier (Model number FD 500/60, Birchover Instruments Ltd). After lyophilisation mycelial samples were crushed with forceps and wood samples were individually milled through a 0.5 mm mesh in a hammer mill (Micro Hammer Mill C580, Glen Creston,

UK) prior to storage at -180°C. Thorough cleaning of the mill between each sample avoided contamination of the samples. Lyophilised material was subsequently used as the source of fungal extracts.

5. ANTISERUM PRODUCTION.

5.1 Immunogen preparation and immunisation.

S. lacrymans FPRL 12C standard mycelium (2.3.2) was prepared as an immunogen as described for L. lepideus by Glancy et al., 1989. A whole cell mycelial suspension was prepared by grinding mycelium in a mortar/pestle at room temperature (R/T) with phosphate buffered saline 10mM, pH 7.4 (PBS) (Appendix A, A7) at a concentration of 5 mg/ml. The immunogen was prepared from this suspension by vortexing 1.5 ml with an equal volume of appropriate adjuvant (primary immunisation: Freund's complete adjuvant, Appendix A, A8; secondary immunisation: Freund's incomplete adjuvant, Appendix A, A9) to give a water-in-oil emulsion. 2 mls of this emulsion was injected into 6 subcutaneous dorsal injection sites on day 1 and day 14.

5.2 Antisera.

5.2.1 Preparation of specific antiserum.

The rabbit was bled from a marginal ear vein 10 days after the booster immunisation. 3 bleeds at approximately fortnightly intervals were taken after repeated booster injections. Serum was prepared in the normal way and was stored in either 1 ml or 100 µl aliquots at -20°C.

5.2.2 Commercial antisera.

The following antisera/control sera were obtained from the Scottish Antibody Production Unit.

a) Horse radish peroxidase-labelled anti-rabbit IgG (donkey polyclonal) (secondary antibody, HRP-Ab2).

b) Normal donkey serum (NDS).

c) Normal goat serum (NGS).

d) Normal rabbit serum (NRS).

Alkaline phosphatase-labelled anti-digoxigenin Fab fragments (sheep) were supplied in the DIG Glycan Detection Kit (Appendix A, A10) kindly supplied by Dr. T.E.J. Buultjens, Department of Biological Sciences, Dundee University.

5.3 Absorbtion of antiserum.

Antiserum for use in the dot immunobinding assay was diluted with an appropriate buffer containing either PBS/0.05%T/5NCS or PBS/5B (Appendix A, A11) to twice the final concentration required. It was mixed with an equal volume of the absorbing agent (2 mg/ml), the standard mycelium of a cross reacting fungal organism, which had been previously ground to a slurry in the same dilution buffer using a mortar/pestle. Organisms used in combination with each other were each used at 2 mg/ml. The mixture was incubated for 60 min at R/T with continuous stirring and centrifuged (2,500 rpm, 10 min). The supernatant was removed and used in subsequent experiments.

6. REAGENTS FOR IMMUNOLOGICAL TECHNIQUES.

6.1 Membranes.

The inert membranes (Appendix A, A12) used in the immunological techniques were nitrocellulose and Immobilon. The former was used for the dot immunobinding assay and the latter was used for western blotting.

6.2 Buffers.

A variety of blocking, dilution and wash buffers were used in the immunological techniques. These (Appendix A, A11) were composed of PBS with either non-specific proteins, e.g. newborn calf serum (NCS), normal goat serum (NGS), normal donkey serum (NDS), bovine serum albumin (BSA) and non-fat dried milk (Blotto (B); Johnson et al., 1984), or a non-ionic detergent, Tween 20 (T), in various combinations and percentages. Most were used in the dot immunobinding assay, with only PBS/T/NCS and PBS/B being used in western blotting.

6.3 Chromogenic substrates.

3 chromogenic substrates were used to visualise the bound proteins (Appendix A, A13). These were diaminobenzidine (DAB) (Graham & Karnovsky, 1966), diaminobenzidine enhanced with nickel chloride (DAB/N) (Hsu & Soban, 1982) and bromochloroindolyl phosphate/nitroblue tetrazolium (BCPIP/NBT) (M^{c}Gadey, 1970; Leary, Brigati & Ward, 1983). DAB was used in the dot immunobinding assay and in western blotting; DAB/N was used for the latter technique only; and BCPIP/NBT was used in glycan detection.

7. DOT IMMUNO-BINDING ASSAY.

7.1 Titre assessment.

To determine if antibody (Ab) had been produced to the specific antigen (Ag) the dot immunobinding assay of Towbin & Gordon (1984), as described for use with *L. lepideus* (Glancy *et al.*, 1989), was used to assess the titre of the test antiserum.

7.1.1 Antigen preparation.

Standard mycelium (2.3.2) was ground to a slurry in a mortar/pestle in PBS at a concentration of 25 mg/ml. This was centrifuged at 13,000 rpm for 10 min at R/T. Doubling dilutions of the supernatant were prepared and 2 µl aliquots were dotted onto nitrocellulose membranes. In later assays samples were incubated at 37°C for 10 min prior to the centrifugation stage.

7.1.2 Initial protocol.

The dot immunobinding protocol is detailed in Appendix A, A14. All incubation steps of the procedure were carried out on a rocking platform. Briefly, the protocol involved blocking of the non-specific binding on the nitrocellulose for 60 min with PBS/0.5T/5NCS; followed by a washing stage with PBS/0.05T which preceded incubation for 60 min with Ab1 at the appropriate concentration in PBS/0.05T/5NCS. Washing stages preceded incubation for 60 min at R/T with HRP-Ab2 at a concentration of 1:200 in PBS/0.05T/5NCS followed by further washing stages.

7.1.3 Detection of HRP-Ab 2 binding.

Detection of the Ab:Ag complex was with DAB. The dots produced in the antigen dilution assays were scored on a visual colour scale from 0 (no colour) to 5, a positive reaction was defined as a score of 2.5 and above.

7.1.4 Non-specific binding to nitrocellulose.

To reduce non-specific binding 2 protocols were investigated. Alternative buffers (Appendix A, A11) were included in the protocol and an extra blocking stage prior to the use of HRP-Ab2 was investigated.

7.1.5 Binding of control antiserum to antigen.

To investigate the apparent binding of control antiserum to antigen 4 systems were tested.

1) Absorbtion of control antiserum with S. lacrymans FPRL 12C standard mycelium.
2) Blocking with sugars - 5 sugars (Appendix A, A15) were included separately in block, dilution and wash buffers at a final concentration of 0.1M in order to bind with possible fungal lectins.
3) Endogenous peroxidase in fungal samples was quenched using the method after Johnstone & Thorpe (1982) (Appendix A, A16).
4) Omission of Ab1 from the protocol.

7.1.6 The effect of sample preparation.

The effect of sample preparation on the non-specific binding of Ab2 was investigated in 2 systems.

1) Detergent treatment using either an ionic detergent, sodium dodecylsulphate (SDS), or non-ionic detergents, e.g. T, Nonidet P40 (NP 40) and

Triton X-100, (Appendix A, A17) in the initial sample extraction buffer.

2) Effect of heat treatment - antigen grinding was followed by incubation at either R/T and 37°C (10 min); or 100°C (3 min).

7.2 Cross-reactivity.

Using the protocol outlined in 7.1.2 a number of fungi (Table 2) were tested for cross reactivity with the antiserum. The antigen was prepared at 25 mg/ml in PBS. In order to improve specificity of the antiserum it was absorbed (5.4) at 1:400 with the most strongly cross reacting fungal species (either S. lacrymans, P. placenta, C. puteana, C. versicolor and S. sanguinolentum, or a combination of C. versicolor and C. puteana) prior to its use in the dot immunobinding assay.

8. PROTEIN SEPARATION BY ELECTROPHORESIS.

8.1 Sample preparation prior to electrophoresis.

8.1.1 Initial experiments.

Weighed samples of lyophilised standard mycelium were ground to a slurry in PBS at R/T using a mortar/pestle. Subsequent addition of 1 part boiling mix (Marsden et al., 1978) (Appendix A, A18) to 2 parts sample was followed by storage at -20°C until use. Mycelial samples were initially prepared at a concentration of 6.25 mg/ml in PBS; wood grown material was prepared at 25 mg/ml in PBS, except for severely decayed material (>50% weight loss) which was prepared at 50 mg/ml.

8.1.2 Efficacy of extraction

The efficacy of the method of sample preparation in extraction of protein was investigated by a variety of methods, e.g. grinding of mycelium (see Appendix A, A19) and variation of extraction protocol (see Appendix A, A20) which included the use of an inhibitor of proteolysis, phenylmethyl-sulphonyl fluoride (PMSF) (Appendix A, A21) in the sample buffer.

8.1.3. Routine sample preparation.

In subsequent experiments weighed samples were ground, at the final concentration of the fungus in PBS, in a cooled mortar/pestle (4°C) in a 2:1 mixture of PBS:boiling mix at 4°C. This was followed by incubation at 100°C for 3 min prior to centrifugation (10 min, 13,000 rpm) and removal of the supernatant (Appendix A, A20, method 6). In the case of some severely decayed wood blocks it was necessary to adjust the pH of the final extract to approximately 6.7 by addition of excess boiling mix. All supernatant samples were stored at -20°C until use.

8.2 Estimation of the protein content of fungal extract.

Extracts of fungal organisms were prepared as in 8.1.1 and the protein content of the supernatant was estimated after centrifugation (13,000 rpm, 10 min) using a dye binding micro-assay based on the method described by Bradford (1976) using Coomassie reagent (Appendix A, A22). Bovine serum albumin (BSA) (Appendix A, A23) was used as the standard protein.

8.3 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE).

8.3.1 Preparation of gels.

Reagents are listed in Appendix A, A24. The proteins in the extracts were analysed by SDS-PAGE using the method of Laemmli (1970) as modified by Marsden et al. (1978). Briefly, this involved the preparation of a 5-15% gradient gel (Appendix A, A25) prepared as described by Marsden et al. (1978) and subsequent addition of a stacking gel (Appendix A, A25).

8.3.2 Sample preparation for SDS-PAGE.

Samples were thawed at R/T, heated at 100°C for 3 min and centrifuged at 13,000 rpm for 10 min at R/T. The supernatant was appropriately diluted in PBS/boiling mix (2:1) and was used in 20 µl samples for analysis by SDS-PAGE. Loading of samples was based on previous experience of the optimum number of bands visualised on stained gels.

8.3.3 Protein standards.

Low (LMW) and high (HMW) molecular weight standard proteins (Appendix A, A26) were thawed prior to use of 20 µl per sample well at either end of gels.

8.3.4 Protein separation.

Proteins in the samples were separated for approximately 4 h at 35 mA/gel at 4°C on a LKB 2001 vertical electrophoresis unit. Following electrophoresis the samples were either stained in the gel or electrophoretically transferred to an inert membrane and subsequently visualised.

9. STAINING OF GEL.

Reagents are listed in Appendix A, A27.

9.1 Protein detection.

Detection of proteins in the gel was by silver staining according to the method of Blum, Beier & Gross (1987) which consists of overnight gel fixation after electrophoresis; pretreatment with sodium thiosulphate; wash with ethanol; impregnation with silver nitrate and formaldehyde; and development with a solution containing sodium carbonate, formaldehyde and sodium thiosulphate. Colour development was stopped when the brown/yellow protein bands were judged to be sufficiently dark. Gels were stored either by drying on a LKB 2003 gel slab drier or in u-p water in sealed polythene bags.

9.2 Calculation of the MW of the proteins.

The relative mobility (Rf value) of each standard protein was calculated using a modification (Sigma Technical Bulletin MWS-877L) of the method of Laemmli (1970).

10. PROTEIN BLOTTING.

SDS-PAGE separated proteins and glycoproteins were transferred (blotted) onto Immobilon using a discontinuous buffer system (Appendix A, A28.1) in the Sartoblot II semi-dry electrophoretic transfer system (Sartorius Ltd) (Appendix A, A28.2) based on a modification (Kyhse-Andersen, 1984) of the method introduced by Towbin, Staehelin & Gordon (1979).

11. MEMBRANE PROBING.

After blotting the membrane was probed for the presence of either proteins, antigens or glycoproteins. All these probing techniques were followed by drying of the membrane at 30°C.

11.1 Protein detection.

The membrane was x2 washed in PBS/0.05T and the method of Hancock & Tsang (1983) was used to detect proteins on the membrane. This involved overnight incubation with constant rocking at R/T with 0.0001% (v/v) India ink solution (Appendix A, A29). This procedure was undertaken on standard protein tracks of all gels to check for efficient protein transfer.

11.2 Antigen detection.

11.2.1 Initial protocol.

The protocol for the treatment of Immobilon carrying blotted fungal antigens initially was the method previously described (7.1.2) for use in the dot immunobinding assay.

11.2.2 Improvement of definition of blot.

To improve the definition of the visualised antigens a number of parameters were investigated (Appendix A, A30), i.e. the method of sample preparation, membranes, buffers which included T, NCS and B; and the concentration of the immunological reagents.

11.2.3 Final protocol.

Samples were prepared as described in 8.1.3 (Appendix A, A20, method 6) and proteins were blotted onto Immobilon using the Sartoblot II transfer apparatus. PBS/T/NCS was routinely used in the buffers (Appendix A, A11) and all incubation steps were carried out on a rocking platform. The non-specific protein binding sites were blocked for 60 min at 4°C using PBS/0.5T/10NCS, washed x2 with PBS/0.05T and incubated overnight at 4°C with *S. lacrymans* FPRL 12C antiserum diluted 1:1600 (v/v) in PBS/0.05T/5NCS; followed by 5 washes with PBS/0.05T and incubation at R/T for 60 min with HRP-Ab2 diluted 1:200 (v/v) in PBS/0.05T/5NCS. Six washes with PBS/0.05T and 2 washes with PBS preceded detection of enzyme activity.

11.2.4 Visualisation of antigens.

2 chromogenic substrates were investigated for the visualisation of blotted antigens (Appendix A, A13). Initially DAB was used but latterly DAB/N was used since its reaction product gives a better contrast for photographic purposes. 2 washing stages with Tris 50mM, pH 7.6 preceded the use of DAB/N. In both cases the colour development was stopped with a final PBS wash prior to drying the Immobilon.

11.3 Glycoprotein detection.

2 different methods were used to investigate the carbohydrate nature of blotted proteins.

11.3.1 Enzyme immunoanalysis.

Glycan detection used the DIG glycan detection kit (Appendix A, A10 and A31). The manufacturer's instructions were followed for the DIG labelling of

glycoproteins immobilised on a membrane. A DIG specific antibody and BCPIP/NBT (Appendix A, A13) visualised these glycoproteins.

11.3.2 Peroxidase labelled lectins.

Blotted samples were reacted with peroxidase labelled lectins, Concanavalin A (Con A) and wheat germ agglutinin (WGA) (Appendix A, A32.1), to localise antigens bearing specific sugar residues. The method used was a modification of that described in Kijimoto-Ochiai, Katagiri & Ochiai (1985) and the appropriate buffers are detailed in Appendix A, A32.2. After blotting the membrane was washed, blocked overnight at 4°C, washed twice prior to reaction with the lectin solution for 60 min at R/T. Final washing stages preceded the detection of enzyme, and hence lectin, binding with DAB/N.

12. ANALYSIS OF GELS AND BLOTS.

12.1 Silver stained gels.

2 methods were assessed for comparative analysis of individual tracks in the stained gels.

12.1.1 Laser densitometry.

The absorbances of the bands in each track were obtained by using the LKB Ultroscan Laser Densitometer 2202.

12.1.2 Visual survey.

Two stages were involved in this method of analysis of stained gels.

12.1.2.1 Maps.

A map was constructed of the protein pattern produced for S. lacrymans FPRL 12C standard mycelium and was based on a visual survey of 6 separate analyses. The staining intensity of each protein band was recorded on a scale of 1 (low intensity) - 3; and the MW of each band was determined. Similar maps were constructed for other isolates to allow a comparison of banding patterns between isolates.

12.1.2.2 Protein percentage similarity.

The relatedness of a test fungal isolate to the reference isolate, usually standard mycelium of S. lacrymans FPRL 12C, was determined by its protein percentage similarity which was based on the number of common bands between the reference and the test isolate. The total number of protein bands on each track was counted and the number of bands common to the test pattern and reference pattern was determined. The resulting number was termed the protein percentage similarity of the test organism and was only estimated when more than 20 bands could be identified for both the test and the reference isolate. Protein percentage similarities were collated into a scale, the protein similarity index, which indicated the range of protein percentage similarities shown by different isolates to the reference isolate.

12.2 Immunostained profiles.

Investigation of immunostained profiles involved

12.2.1 Antigenic profile of S. lacrymans FPRL 12C.

2 bands at approximately 180 kilo-Daltons (kDa) and 12 kDa were regularly observed in the antigenic profile of S. lacrymans FPRL 12C standard mycelium. These bands were used as the framework within which the positions of the standard proteins and antigenic species in 25 separate analyses of S. lacrymans FPRL 12C standard mycelium were calculated. This resulted initially in discrimination of a total of 19 antigenic species in the profile of S. lacrymans FPRL 12C standard mycelium which were sequentially numbered 1 (180 kDa) - 19. Later analyses revealed that antigens 4 and 5 were regularly composed of 2 bands so these were incorporated into the reference antigenic profile of S. lacrymans as antigens 4.25 and 5.25. The approximate molecular weight and the percentage occurrence of each of these antigens in the 25 analyses was calculated.

12.2.2 Antigenic percentage similarity.

The relatedness of each test sample to the standard preparation of S. lacrymans FPRL 12C was determined by its antigenic percentage similarity which was obtained by comparison of a test profile with the 21 antigens in the reference profile established for FPRL 12C. Antigenic percentage similarities were calculated when 10 antigenic species could be identified for both the test and the reference isolate. Antigenic percentage similarities were collated into a scale, the antigenic percentage similarity index, which indicated the range

of antigenic percentage similarities shown by different isolates to the reference isolate.

12.2.3 Diagnostic antigens.

Identification of the diagnostic antigens which were characteristic of each field morphological form was undertaken by analysis of tracks which displayed the greatest number of antigenic species. An antigen which featured in >70% of the tracks analysed was termed a diagnostic antigen. Diagnostic antigens for each morphological form were compiled into the diagnostic antigenic profile which formed the basis for comparison between morphological forms of S. lacrymans. Antigenic species which were present in the field morphological forms but were not present in the reference track of S. lacrymans FPRL 12C standard mycelium were allocated an antigenic number which represented their position in relation to two established antigenic species in the reference track. Samples analysed for the production of the appropriate diagnostic antigenic profile for each form were: mycelium - 10 samples from site A in 3 analyses; basidiocarp - 3 samples from site C in 2 analyses; and strand - 1 sample from site A in 4 analyses.

CHAPTER 3. THE MOLECULAR PROFILE OF *S. lacrymans*.

3.1 General introduction.

SDS-PAGE/silver staining, lectin staining and western blotting were used to elucidate the nature of the molecular profile of a PBS soluble extract from standard mycelium of *S. lacrymans*. Preliminary studies concentrated on the isolate chosen as the reference culture, FPRL 12C, against which comparisons of other isolates were made. Immunological studies were initiated using the dot immunobinding assay to investigate the nature of the interaction of the antiserum raised against standard mycelium of *S. lacrymans* FPRL 12C with a PBS extract of the antigen.

The main objectives of the work reported in this chapter were

1. To establish a reference protein profile for a PBS soluble extract of standard mycelium of *S. lacrymans* FRPL 12C.
2. To extend the studies detailed in 1. to other *S. lacrymans* isolates.
3. To develop an immunological probe for *S. lacrymans*.
4. To establish a reference antigenic profile for the PBS soluble extract of standard mycelium of *S. lacrymans* FPRL 12C.
5. To extend the studies detailed in 4. to other *S. lacrymans* isolates.
6. To compare glycoprotein profiles of standard mycelium of *S. lacrymans* with the profiles obtained in 2 and 5.

3.2 The protein profile of S. lacrymans.

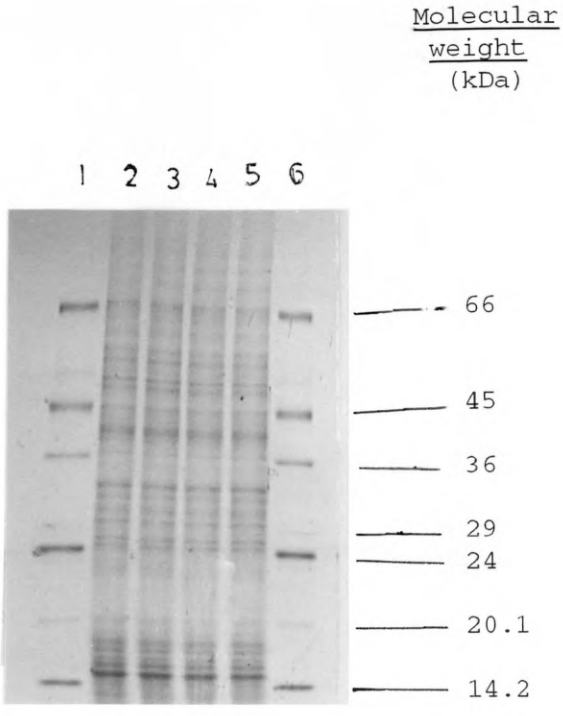
3.2.1 The profile of S. lacrymans FPRL 12C.

The molecular profile of S. lacrymans FPRL 12C was investigated after 6 separate analyses. Protein patterns varied slightly in successive gels in that not all bands were equally well resolved on each occasion. However, all tracks were visually similar and the results for one experiment are shown in Figure 5. These results showed that PBS soluble extract from the standard mycelium of S. lacrymans FPRL 12C was composed of 30 - 50 protein bands whose molecular weights ranged from 13 - 160 kDa. The definition and intensity of these proteins varied within a track, especially those proteins >66 kDa, but the disposition of these major and minor proteins contributed to the impression of visual similarity between tracks. The tracks analysed had at least 29 protein bands in common and formed a recognisable pattern which was seen to be reproducible even prior to more detailed analysis. The protein pattern for mycelial extracts from standard mycelium of S. lacrymans FPRL 12C was represented as a map (Figure 6) to which the molecular weight of each protein band has been added.

3.2.2 The profile of other S. lacrymans isolates.

A standard mycelial extract from a collection of isolates of S. lacrymans was analysed and compared with the standard preparation of S. lacrymans FPRL 12C in order to determine if the profile established for S. lacrymans FPRL 12C was unique to that isolate. The results for one experiment are shown in Figure 7 and a representative map of the arrangement of the protein

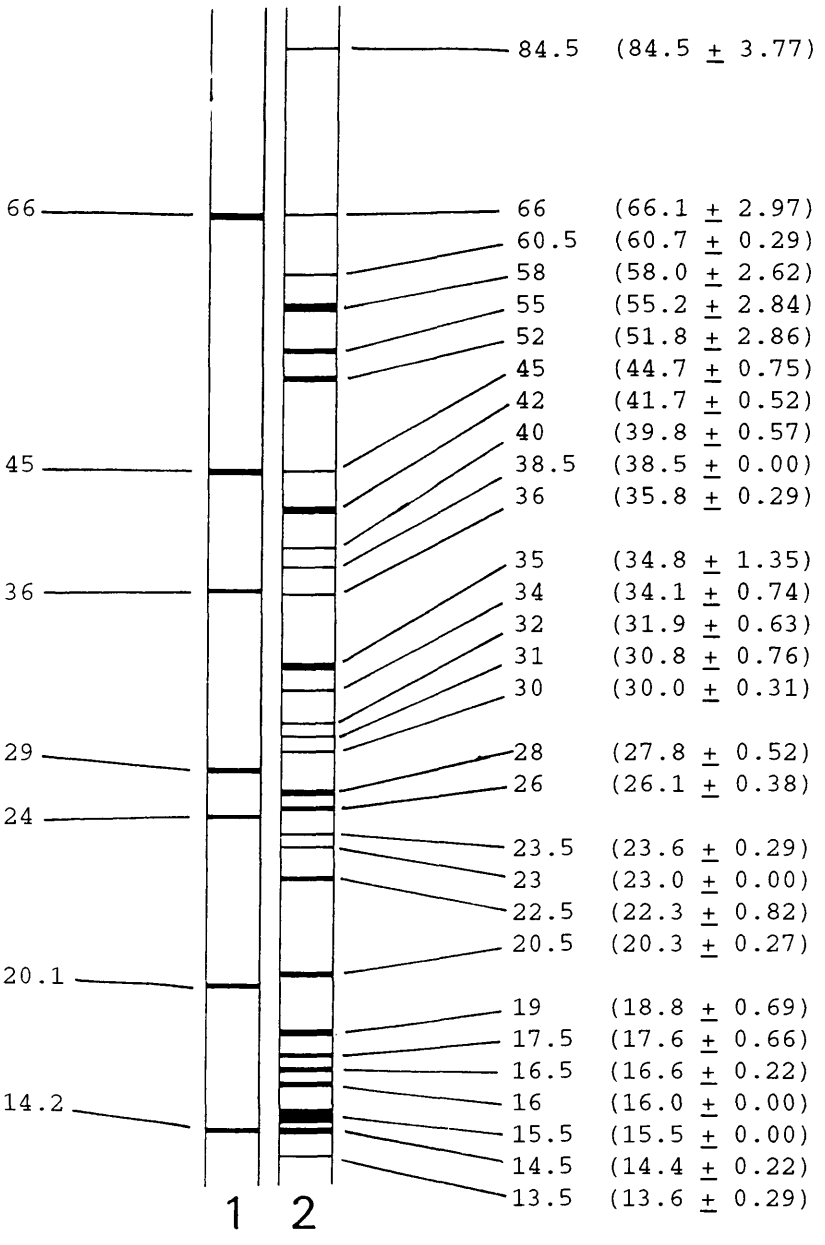
Figure 5: SDS-PAGE/silver stain analysis of standard mycelial extract of S. lacrymans FPRL 12C.



Tracks 1 and 6 represent molecular weight standard proteins.

Tracks 2 - 5 represent analyses of 4 different extracts of standard mycelium of S. lacrymans FPRL 12C.

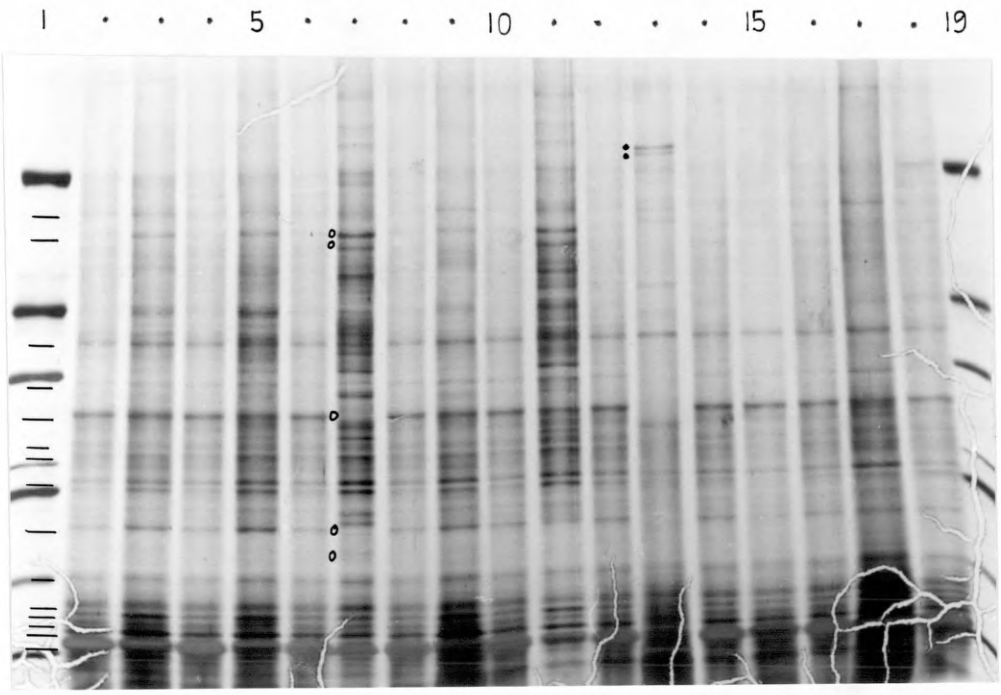
Figure 6 : Diagrammatic representation of the bands associated with the standard mycelium of S. lacrymans FPRL 12C and their respective molecular weights (kDa) calculated from an analysis of the tracks of 6 different extracts of standard mycelium of S. lacrymans FPRL 12C.



Track 1 represents molecular weight standard proteins. Track 2 represents S. lacrymans FPRL 12C standard mycelium.
Figures in parenthesis indicate the arithmetical mean and sample standard deviation of 6 analyses.

Band intensity Minor proteins: ——— least intensely stained.
Major proteins: ——— medium staining intensity.
▬▬▬ most intensely stained.

Figure 7: SDS-PAGE/silver stain analysis of various isolates of S. lacrymans.



The molecular weight markers shown in tracks 1 and 19 were of the following sizes : 14.2, 20.1, 24, 29, 36, 45 and 66 kDa.
S. lacrymans FPRL 12C is shown in tracks 2, 4, 6, 8, 10, 12, 14, 16 and 18.
Other S. lacrymans isolates are in tracks 3 (BF-023); 5 (BF-01); 7 (BF-050); 9 (BF-07B); 11 (BF-017B); 13 (BF-015B); 15 (CMI 152233)) and 17 (BF-03A).

__ represents bands found in most isolates.
o represents differences between BF-050 and FPRL 12C.
. represents bands unique to BF-015B.

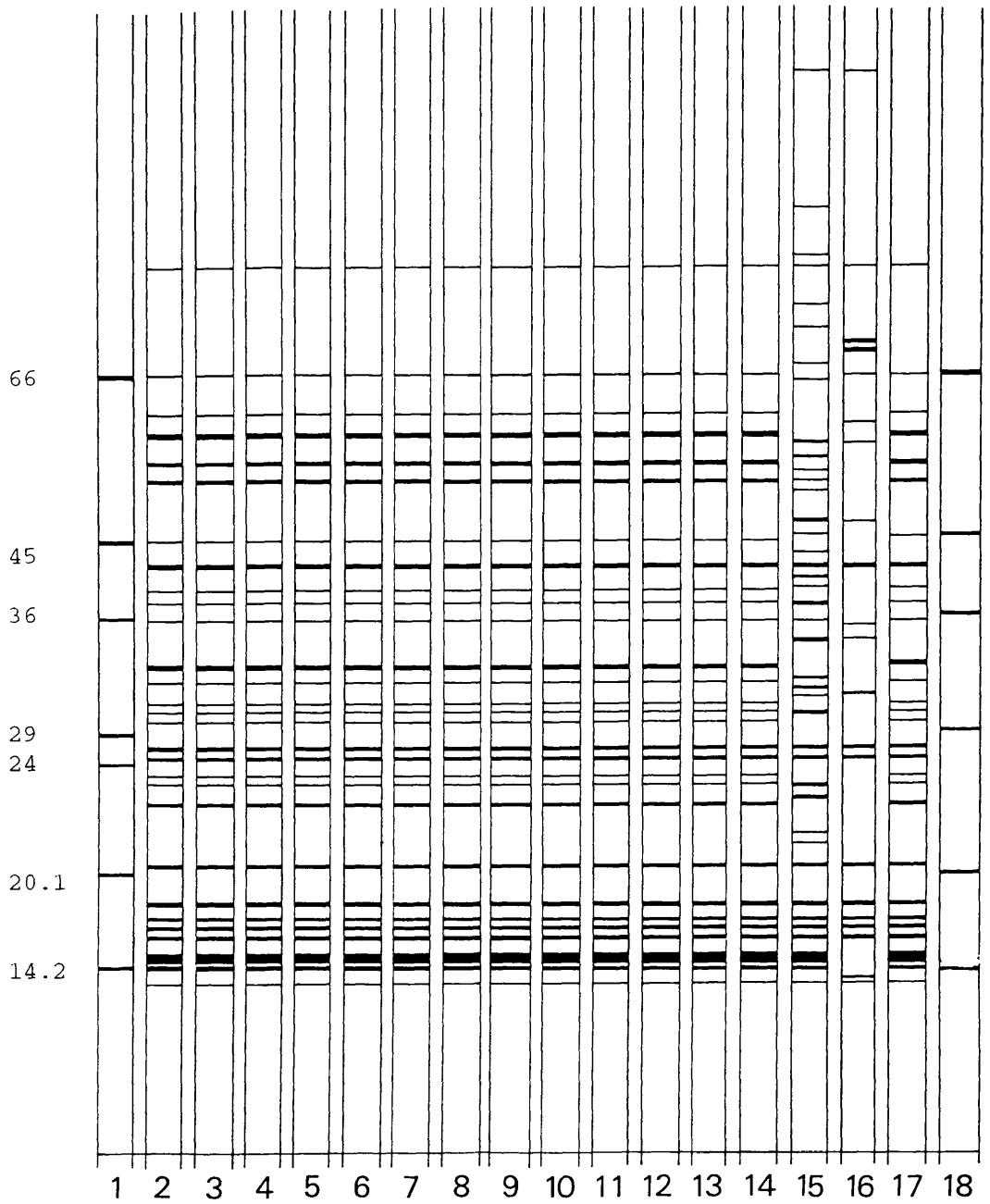
bands in S. lacrymans isolates is shown in Figure 8. For ease of comparison the distribution of the major proteins in the profiles are presented in Table 5. The data indicate that the majority of isolates, regardless of the date of original isolation or the geographical location of the isolate source, show a protein banding pattern which is visually similar to S. lacrymans FPRL 12C since major proteins at 16, 16.5, 17.5, 19, 20.5, 26, 28, 32.5 and 42 kDa are common to all isolates. Additionally, major proteins at 14.5, 15.5, 22.5, 23, 31, 35, 38.5, 52, 55 and 58 kDa are common to most isolates.

Even though minor differences can be observed between some tracks on Figure 7 the over-riding visual impression is that only 2 isolates, BF-050 (track 7) and BF-015B (track 13) have profiles which, whilst showing similarities to 12C, differ noticeably from that of S. lacrymans FPRL 12C. This is confirmed by Table 5 which indicates that BF-015B is least similar to FPRL 12C. BF-050 differs from FPRL 12C in that the major proteins at 22.5, 35, 55 and 58 kDa are absent and there are major proteins at 55.5 and 57 kDa which are not present in FPRL 12C. Amongst the differences between FPRL 12C and BF-015B are bands of molecular weight of 72 and 74 kDa (present only in BF-015B) and 14.5, 15.5, 22.5, 35, 52, 55 and 58 kDa (present only in FPRL 12C).

3.2.3 Discussion.

SDS-PAGE/silver staining is a valuable technique which has been widely used in mycology for the analysis of

Figure 8: Diagrammatic representation of the bands associated with a range of different isolates of S. lacrymans.



Molecular weight markers (kDa) are shown in tracks 1 and 18.
S. lacrymans FPRL 12C is represented in tracks 2 and 17.
Other S. lacrymans isolates are shown in tracks 3 (CMI 152233); 4 (BF-01); 5 (BF-03A); 6 (BF-07B); 7 (BF-017B); 8 (BF-018A); 9 (BF-023); 10 (BF-025); 11 (BF-044); 12 (BF-046); 13 (BF-049); 14 (BF-072); 15 (BF-050)) and 16 (BF-015B).

Major proteins: ▬ most intensely stained.
▬ medium staining intensity.
Minor proteins: ——— least intensely stained.

Table 5: Major protein bands in S. lacrymans isolates after SDS-PAGE/silver stain.

Molecular weight (kDa)	isolate FPRL 12C	isolate BF-050	isolate BF-015B	other isolates
74	-	-	++	-
72	-	-	++	-
58	++	-	-	++
57	-	++	+	-
55.5	-	++	-	-
55	++	-	-	++
52	++	+	-	++
46	-	++	+	-
42	++	++	++	++
38.5	+	++	-	+
35.7	-	++	+	-
35	++	-	-	++
34.5	-	++	-	-
33	-	++	-	-
32.5	+	+	++	+
31	+	++	-	+
28	++	++	++	++
26	++	++	++	++
23	+	++	-	+
22.7	-	++	-	-
22.5	++	-	-	++
20.5	++	++	++	++
19	++	++	++	++
17.5	++	++	++	++
16.5	++	++	++	++
16	++	++	++	++
15.5	+++	+++	-	+++
14.5	++	++	-	++

+++ indicates major proteins of greatest staining intensity.
++ indicates major proteins of intermediate staining intensity.
+ indicates minor proteins which stained least.
- indicates no staining detectable.

complex mixtures of proteins which are found in extracts of fungal organisms. Wood decay Basidiomycetes such as H. annosum (Palfreyman et al., 1990), S. lacrymans (Schmidt & Kebernik, 1989), L. lepideus (Glancy, 1990) and C. puteana (M^{c}Dowell & Palfreyman, 1992), have been analysed by SDS-PAGE/silver staining. Not only can information on the molecular weight of specific proteins be estimated but similarities and differences between different extracts can be determined by comparison of banding patterns. The technique has been used in this project to demonstate that the majority of S. lacrymans isolates share a similar molecular profile when grown under identical conditions and these conclusions are in accord with those of Schmidt & Kebernik (1989). That different isolates of other fungal species share a similar molecular profile when analysed by SDS-PAGE/silver staining has also been demonstrated in other Basidiomycetes, e.g. G. trabeum (Palfreyman et al., 1990), C. puteana (M^{c}Dowell & Palfreyman, 1992) and H. annosum (Galbraith, personal communication); and for other fungal organisms, e.g. Sclerotinia (Jellis, Smith & Scott, 1990); and Gaeumannomyces graminis var tritici and Phialophora spp (Maas et al., 1990).

Since SDS-PAGE/silver staining has indicated similarities and dissimilarities between isolates of S. lacrymans it would appear that the technique has potential not just for the study of the protein nature of S. lacrymans but also for organism identification. The dissimilarity of protein profile shown by two isolates of S. lacrymans possibly indicates that these

organisms were mistakenly identified as S. lacrymans but, as yet, no obvious differences in morphological features of the isolates, their place of isolation or their growth rate (Cymorek & Hegarty, 1986) have been established to further differentiate them from the other isolates of S. lacrymans. However, Schmidt & Kebernik (1989), using SDS-PAGE/silver stain backed up by growth studies, have indicated that BF-015B is not an isolate of S. lacrymans but rather a taxonomically closely related species.

The possession of common proteins could suggest either a close taxonomic relationship or that these proteins are ubiquitous fungal proteins. Interestingly, in spite of the difference between isolate FPRL 12C and BF-050 which was indicated by protein profiles, Schmidt (personal communication) was convinced that BF-050 was an isolate of S. lacrymans; with the implication that some variation in protein profile was to be expected between isolates of a species. This implication is illustrated in the project by minor differences seen between the isolates which most closely resemble the reference isolate, FPRL 12C. These results are in accord with studies on G. trabeum and H.annosum (Palfreyman et al., 1990), C. puteana (M^{c}Dowell & Palfreyman, 1992), ; and non-Basidiomycetes such as Sclerotinia (Jellis, Smith & Scott, 1990) and Aspergillus fumigatus (Hearn et al., 1990) isolates and illustrate that strains of the same species vary within cultural limits (Foster, 1949).

3.3 Development of an immunological probe for S. lacrymans.

3.3.1 Initial dot immunobinding assay.

The immunodot assay was used to determine if antibodies had been produced to the specific antigen. Results indicated that the reactivity of the specific antiserum, diluted 1:400, declined below an antigen concentration of 1.56 mg/ml since this was the lowest concentration at which a positive reaction of 4 was scored in the primary antiserum dilution assay. However, since there was a high degree of non-specific binding of antiserum to the nitrocellulose alternative buffers were investigated to overcome this problem. It was found that PBS/B eliminated the non-specific binding of antiserum to the membrane and was adopted as the standard buffer in subsequent immunodot assays.

There was also an apparent reaction between S. lacrymans and the control serum (Figure 9A, tracks 1 - 3), even at the lowest concentration of the antigen (Figure 9A, track 1), which was not observed in the other fungal organisms (tracks 4 - 17). However, analysis of this reaction indicated that the second antibody, the peroxidase linked anti-rabbit IgG, was binding to the antigen. A variety of procedures were investigated to reduce this binding, including an analysis of the effect of ionic and non-ionic detergents in sample preparation. Ionic detergents, especially SDS, markedly reduced the binding of the second antibody to the antigen and eliminated the apparent reaction of control serum with antigen.

Figure 9 : The use of the dot immunobinding assay to investigate the cross reactivity of the antiserum to S. lacrymans FPRL 12C.

Figure 9A.

NRS used as Ab1 at 1:400.

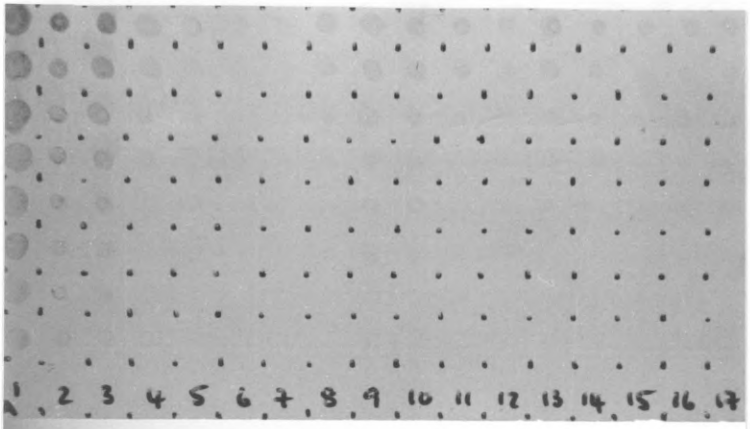
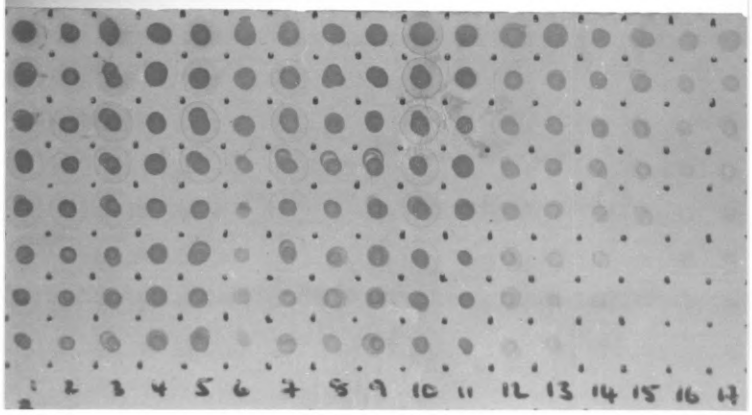


Figure 9B.

Antiserum to S. lacrymans FPRL 12C used as Ab1 at 1:400.



Fungal antigens were prepared from an initial 25 mg/ml (top row of tracks 1 - 17) in 8 doubling dilutions in PBS. The fungal organisms represented in tracks 1 - 17 are as follows : S. lacrymans FPRL 12C (1); S. lacrymans CMI 152233 (2); S. lacrymans BF-01 (3); P. placenta (4); C. puteana (5); G. sepiarium (6); G. trabeum (7); L. lepideus (8); C. versicolor (9); S. sanguinolentum (10); H. annosum (11); S. commune (12); P. ostreatus (13); C. resinae (14); P. variotti (15); T. polysporum (16); and T. harzianum (17).

The immunodot assay was used to investigate the reactivity of the antiserum to fungal organisms other than S. lacrymans FPRL 12C. S. lacrymans isolates CMI 152233 and BF-01 reacted with the antiserum to isolate FPRL 12C (Figure 9B, tracks 2 and 3) but marked cross reactivity was observed with the other fungi investigated (Figure 9B, tracks 4 - 17) although S. commune, P. ostreatus and the non-Basidiomycetes (Figure 9B, tracks 12 - 17) had limited cross reactivity at lower concentrations of the antigen. In an attempt to reduce this cross reactivity alternative preparation methods of the antigen were investigated. Marked cross reactivity was observed with all the other fungi when the soluble antigen extract was prepared in Tween but treatment with SDS improved the specificity of the antiserum for S. lacrymans in that little cross reactivity was observed with any of the other fungi. However, treatment with either detergent resulted in a drastic loss of signal against S. lacrymans.

3.3.2 Discussion.

The reaction of the antiserum to S. lacrymans FPRL 12C with S. lacrymans isolates indicated that the immunisation protocol had produced antibodies that could react with S. lacrymans. However, since there was a high degree of cross reactivity with the other fungal organisms tested it was concluded that the antiserum developed in the project does not show a high specificity for S. lacrymans. The high degree of cross reactivity found with the S. lacrymans antiserum is not surprising given results in other areas of mycology (Pepys & Longbottom, 1979). Since basidiomycete fungi have both common and unique components extensive

antigenic cross reaction could occur and, additionally in support of the results of the present study, it was found that, firstly, the mycelium from P. placenta cross reacted strongly with antisera raised against L. lepideus and C. versicolor (Palfreyman et al., 1987) and, secondly, antiserum raised against mycelium of C. puteana showed considerable cross reactivity (M^{c}Dowell & Palfreyman, 1992). Whilst detergent treatment of the antigen lowered cross reactivity with other fungal organisms it similarly lowered reactivity with the specific antigen so it is probable that the high degree of cross reactivity shown by the test antiserum towards all the other fungi is a representative picture of the true degree of cross reactivity.

The binding of the second antibody to the antigen was presumably non-specific and it is possible that commercial preparations of this serum were made from animals which had been inadvertantly exposed to cross reacting fungal antigens (Pepys & Longbottom, 1979). Interestingly, Palfreyman et al. (1987) noted that a number of different fungi showed some apparent reaction with control sera, the brown rot organism P. placenta being particularly reactive. However, in the present study S. lacrymans was the only species to show this characteristic and, in support of the present findings, M^{c}Dowell & Palfreyman (1992) found that C. puteana did not bind experimental control components. The reduction by SDS of the binding of the second antibody is a factor which undoubtedly aided in subsequent immunoblotting experiments.

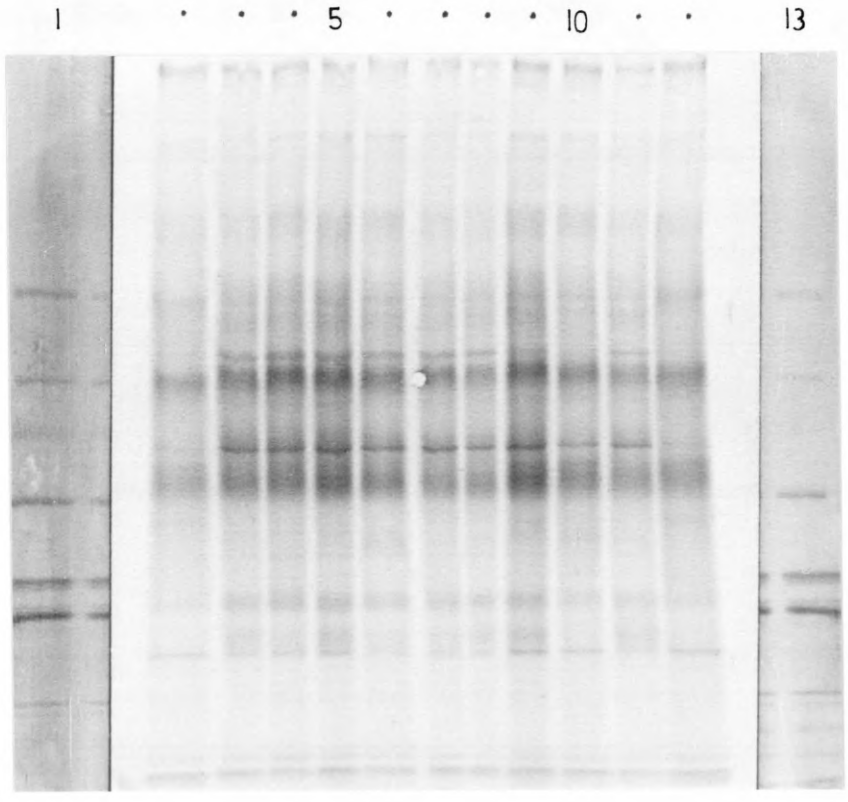
3.4 The antigenic profile of S. lacrymans.

3.4.1 The profile of S. lacrymans FPRL 12C.

Western blotting experiments indicated, firstly, that in this system there was no binding between experimental control components and antigen and, secondly, that the profile was mainly composed of diffuse antigenic bands although occasional sharply defined proteins were detected (Figure 10). Attempts to improve the definition of all bands involved investigation of a number of procedures related to either experimental technique or the preparation of samples. The most successful of these were related to sample preparation and involved either the use of inhibitors of proteolysis or processing of samples in PBS:boiling mix at 4°C followed by heating at 100°C for 3 min (track 7). The latter procedure was used subsequently though the major antigenic bands of S. lacrymans were still diffuse and the overall antigenic profiles remained less well defined than the comparable protein profiles.

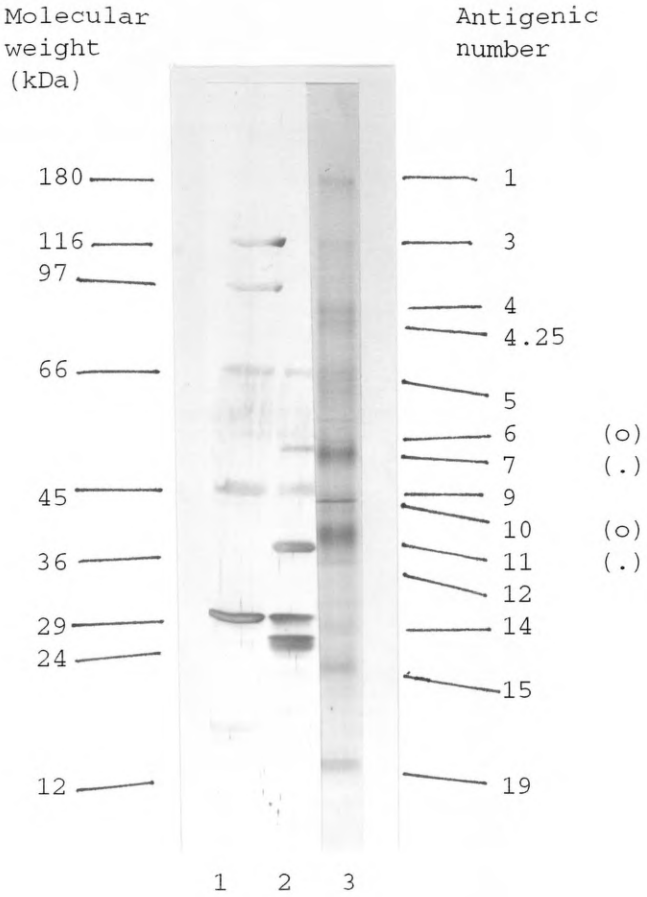
Figure 11 (track 3) illustrates a representative antigenic profile for S. lacrymans FPRL 12C standard mycelium. The antigenic profile of standard mycelial extract of S. lacrymans FPRL 12C was investigated after 25 separate analyses. All tracks were visually similar and each was composed of at least 19 identifiable antigenic regions. Figure 12 shows the antigenic profile associated with S. lacrymans FPRL 12C standard mycelium and the antigenic number (also shown on Figure 11) assigned to each constituent antigen for ease of reference. The approximate molecular weight assigned to

Figure 10: The effect of extraction conditions on the antigenic profile of S. lacrymans FPRL 12C.



Tracks 1 and 13 represent low molecular weight standard proteins.
Tracks 2 and 12 are the standard preparation of S. lacrymans FPRL 12C.
Samples of FPRL 12C in tracks 4, 6, 8 and 10 were extracted at R/T and those in tracks 3, 5, 7, 9 and 11 at 4°C.
Samples in tracks 7, 8, 9 and 10 were extracted directly into boiling mix and either heated at 100°C for 3 min prior to storage at -20°C (7, 8) or stored immediately at -20°C (9, 10).
Samples in track 11 were kept at 4°C until storage at -20°C.

Figure 11: The antigenic profile, and the antigenic number assigned to each antigen, of S. lacrymans FPRL 12C standard mycelium after western blotting using a polyclonal antiserum raised against S. lacrymans FPRL 12C.



Tracks 1 and 2 represent high and low molecular weight standard proteins. Track 3 shows the antigenic profile of S. lacrymans FPRL 12C standard mycelium prepared at a concentration of 6.25 mg/ml.

(o) indicates well defined antigens.
(.) indicates major diffuse antigens.

Figure 12: Diagram of the antigenic profile of S. lacrymans FPRL 12C standard mycelial extract (25 tracks analysed).

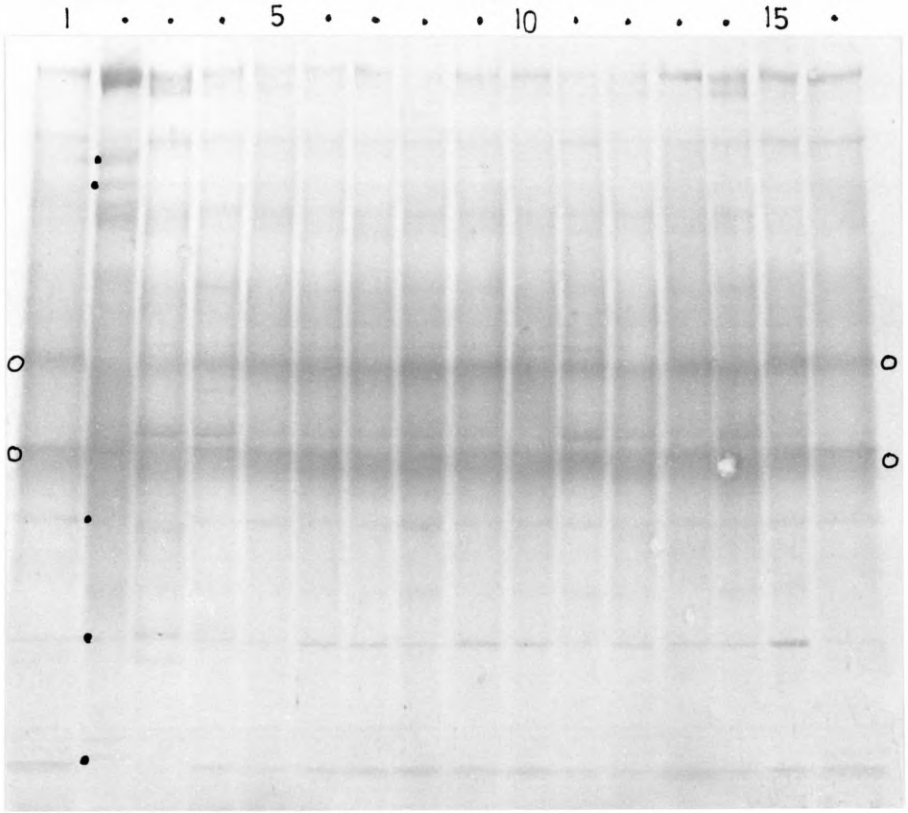
Antigenic profile	Antigenic number	Approximate molecular weight	Percentage occurrence in 25 tracks
_____	1	180	100
_____	2	160	20
_____	3	116	100
_____	4	97.5	100
_____	4.25	95.5	100
_____	5	66	88
_____	5.25	64.5	88
_____	6	57	56
//////////	7	51 - 54.5	96
_____	8	49	44
_____	9	44	48
_____	10	43	84
//////////	11	41.5 - 42.5	100
_____	12	33	80
_____	13	30 - 31	52
_____	14	23.5 - 24.5	72
_____	15	22.5	72
_____	16	19.5 - 20.5	12
_____	17	14	40
_____	18	13	52
_____	19	12	100

each molecular species and its percentage occurrence in the 25 tracks analysed is also shown in Figure 12.

The profile is composed of a variety of major and minor antigenic species whose approximate molecular weights range from 12 to 180 kDa. Most are of a diffuse nature, with 2 notable diffuse major antigens represented at 51 - 54.5 kDa (antigen 7) and 41.5 - 42.5 kDa (antigen 11) but 2 well defined antigens are represented at 57 kDa (antigen 6) and 43 kDa (antigen 10). A well defined antigen (44 kDa, antigen 9) which was represented in some other tracks of S. lacrymans FPRL 12C standard mycelium is not obvious in Figure 11. The profile includes 21 antigens because analyses of recent profiles of S. lacrymans FPRL 12C indicated that antigens 4 and 5 were each regularly composed of two distinct bands. These molecular species were incorporated into the 21 components of the antigenic profile as antigens 4.25 and 5.25. However, only 6 of the total possible antigens were present in the profile of all tracks analysed. These included antigens at the extremes of the molecular weight range (antigens 1 and 19) and one of the diffuse major antigens (antigen 11).

3.4.2 The profile of other S. lacrymans isolates. Standard mycelium from other isolates of S. lacrymans was analysed and compared with the standard preparation of the reference isolate FPRL 12C. The results for one experiment are shown in Figure 13. Comparisons of the antigenic profile of all the S. lacrymans isolates indicated that most isolates visually resembled S. lacrymans FPRL 12C and with one exception, isolate BF-015B (track 2), all isolates (tracks 3 - 15) possessed

Figure 13: The antigenic profile of S. lacrymans isolates.



Tracks 1 and 16 represent extracts of S. lacrymans FPRL 12C.
Tracks 2 - 15 represent the following isolates of S. lacrymans:
BF-015B (2); BF-050 (3); BF-072 (4); BF-049 (5); BF-046 (6); BF-047 (7); BF-025 (8); BF-023 (9); BF-018A (10); BF-017B (11); BF-07B (12); BF-03A (13); BF-01 (14) and CMI 152233 (15).

The bands indicated thus (o) represent major antigens of 41.5 - 42.5 (antigen 11) and 51 - 54.5 (antigen 7) kDa found in S. lacrymans FPRL 12C standard mycelium.

The bands indicated thus (.) represent differences between BF-015B and FPRL 12C.

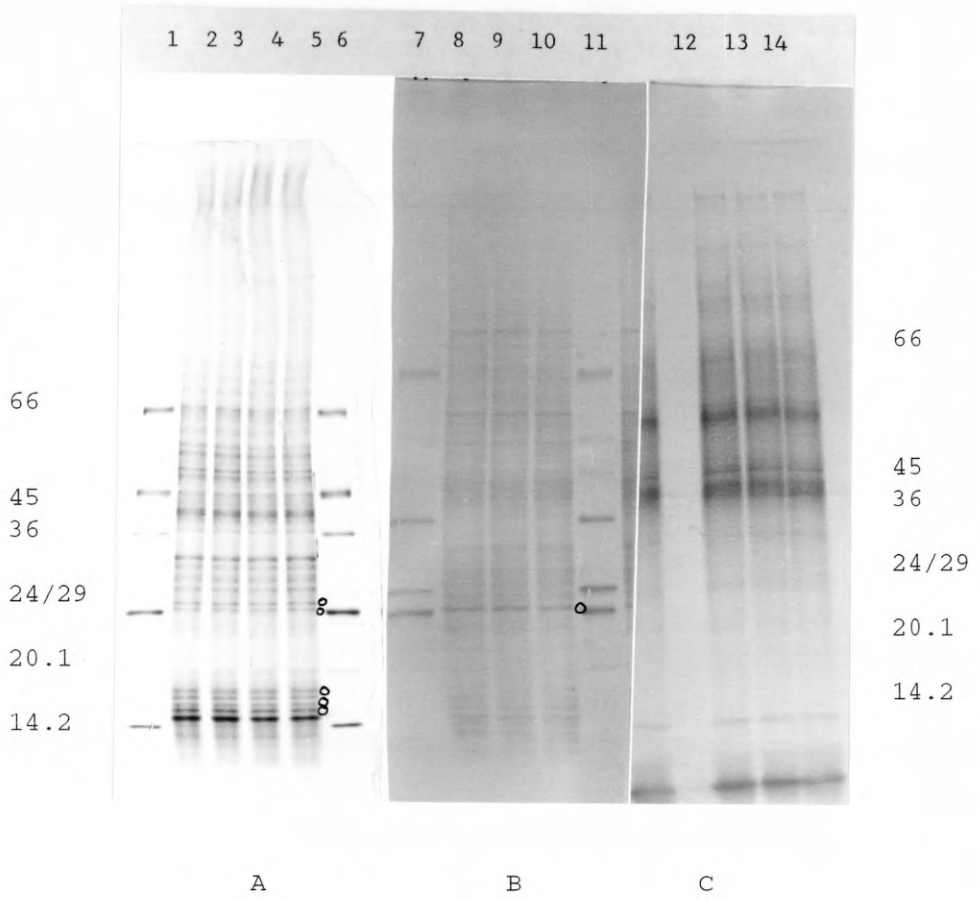
antigens found in the profile of S. lacrymans FPRL 12C (Figure 12) even though differences were observed between individual profiles. Most isolates possessed antigens 1, 3; the major antigens 7 and 11; and 19. The profile of isolate BF-015B did not possess antigens 17 - 19, 15 or 12 and it possessed two antigens between 97.5 - 116 kDa found in no other S. lacrymans isolate (Figure 13, track 2).

The relationship between the antigenic and SDS-PAGE/silver stained profiles of S. lacrymans FPRL 12C was investigated and the results are shown in Figure 14. Table 6 summarises the information presented in Figures 6, 12 and 14. This indicates, firstly, that some proteins are not antigens, e.g. those at 84.5, 60.5, 58, 34 -40 and 14.5 - 19 kDa; and secondly, that antigens 1 - 4.25 (>66 kDa) and the well defined antigens 6, 9 and 10 are proteins which are not easily detected with SDS-PAGE/silver stain. Some antigens which correspond to SDS-PAGE/silver stained proteins are antigens 5 (66 kDa), 13 (30 -31 kDa) and 15 (22.5 kDa). Interestingly, some diffuse antigens, e.g. 14, 16 and the major antigens 7 and 11, each appear to correspond to just one defined protein band on SDS-PAGE/silver stained gels.

3.5 The glycoprotein components of S. lacrymans.

Lectin binding patterns were investigated in order to further analyse the molecular profiles of isolates of S. lacrymans. The results are shown in Figure 15 (WGA) and Figure 16 (Con A) which both indicate a diffuseness

Figure 14: The relationship between SDS-PAGE/silver stained proteins and the antigenic proteins of standard mycelium of S. lacrymans FPRL 12C.



Sections A - C were obtained from one gel. Section A represents SDS-PAGE/silver stained gel; section B represents India ink stained membrane after electroblotting; and section C represents the immunostained portion of the membrane.

Tracks 1, 6, 7 and 11 represent molecular weight standard proteins.

Tracks 2 - 5, 8 - 10 and 12 - 14 represent standard mycelial extract of S. lacrymans FPRL 12C at 6.25 mg/ml.

o indicates proteins which are not antigens.

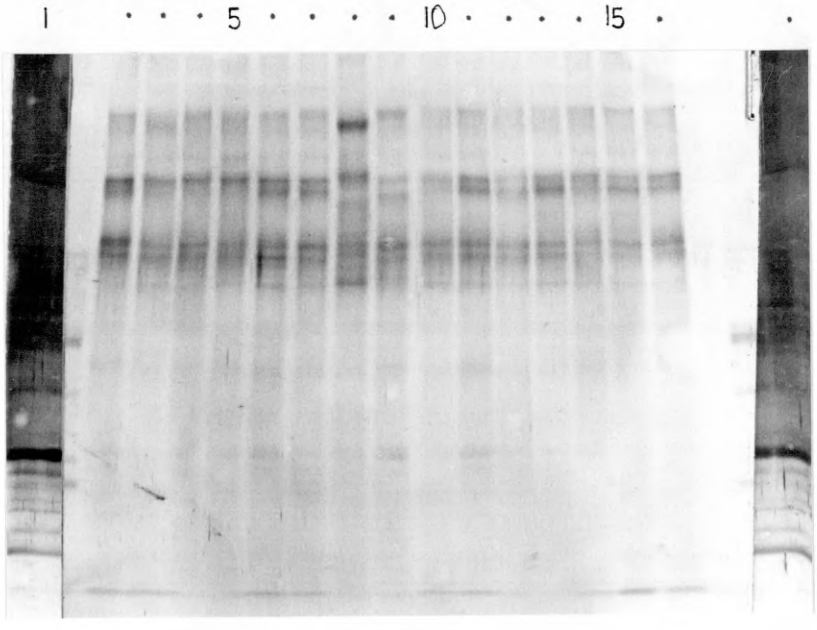
Table 6: Protein identity of antigens identified by western blotting of S. lacrymans FPRL 12C standard mycelium.

Protein regions identified in S. lacrymans FPRL 12C mycelium.

Approximate molecular weight (kDa)	Antigen number	SDS-PAGE/ silver stain	Lectin stain
180	1	-	o
160	2	-	-
116	3	-	-
97.5	4	-	o
95.5	4.25	-	o
84.5	-	+	-
66	5	+	o
64.5	5.25	-	-
58 - 60.5	-	+	-
57	6	-	-
55	-	+	-
54.5	7	-	o
52	7	+	o
51	7	-	o
49	8	-	-
45	-	+	-
44	9	-	-
43	10	-	-
42.5	11	-	?
42	11	+	?
41.5	11	-	?
34 - 40	-	+	-
33	12	-	-
32	-	+	-
31	13	+	-
30	13	+	-
26 - 28	-	+	-
24.5	14	-	-
23.5	14	+	-
23	-	+	-
22.5	15	+	-
20.5	16	+	-
19.5	16	-	-
14.5 - 19	-	+	-
14	17	-	-
13.5	-	+	-
13	18	-	-
12	19	-	-

+ indicates protein demonstrated by technique.
- indicates protein nature not demonstrated by technique.
o indicates glycoprotein demonstrated by technique.
? indicates dubiety about nature of band.

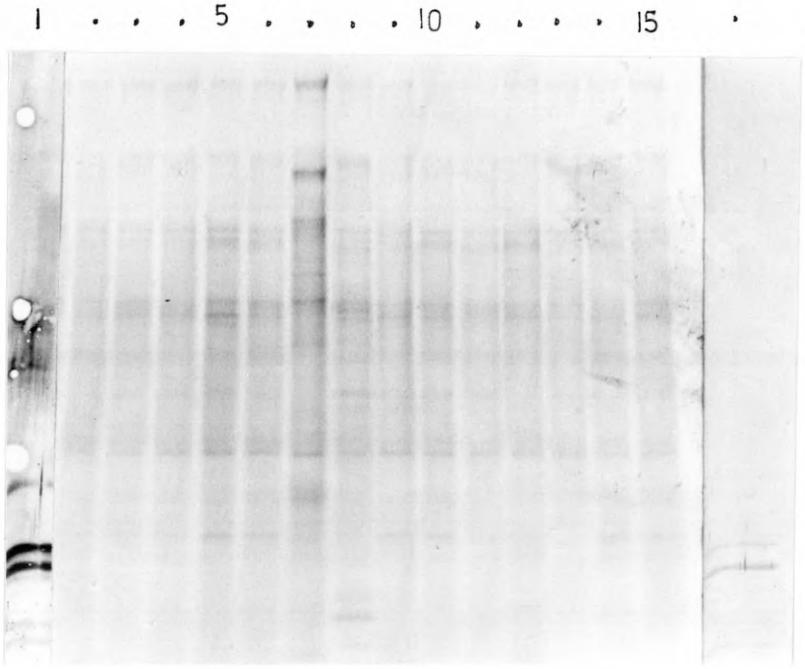
Figure 15: Analysis of the wheat germ agglutinin binding proteins of extracts of standard mycelium from a range of S. lacrymans isolates.



Tracks 1 and 17 represent molecular weight standard proteins.

Tracks 2 - 16 represent the following isolates of S. lacrymans: BF-072 (2); BF-049 (3); BF-046 (4); BF-044 (5); BF-025 (6); BF-023 (7); BF-015B (8); BF-050 (9); BF-018A (10); BF-017B (11); BF-07B (12); BF-03A (13); BF-01 (14); CMI 152233 (15) and FPRL 12C (16).

Figure 16: Analysis of the Concanavalin A binding proteins of extracts of standard mycelium of S. lacrymans isolates.



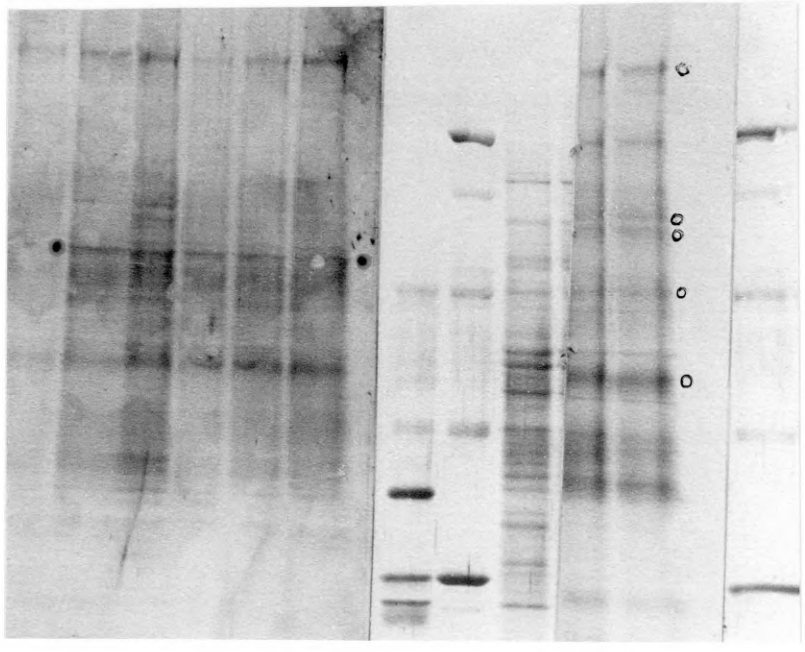
Tracks 1 and 16 represent molecular weight standard proteins.
Tracks 2 - 15 represent the following isolates of S. lacrymans: BF-072 (2); BF-049 (3); BF-044 (4); BF-025 (5); BF-023 (6); BF-015B (7); BF-050 (8); BF-018A (9); BF-017B (10); BF-07B (11); BF-03A (12); BF-01 (13); CMI 152233 (14) and FPRL 12C (15).

in the staining pattern associated with each glycoprotein. The pattern of binding with each lectin differs but each indicates that there are minor differences between most isolates. Essentially, however, all isolates except one, BF-015B, have similar glycoprotein profiles. The result of an investigation into the glycoprotein nature of the antigens of S. lacrymans are shown in Figure 17 which indicates that, in spite of the fact that only glycoproteins >36 kDa stained, some of the antigens are glycoproteins, e.g. notably antigens 1, 4, 4.25, 5, diffuse major antigen 7 and, possibly, 11. Whilst some antigens are unlikely to be glycoproteins, e.g. antigens 3, 6 and 8, other glycoproteins are not antigens, e.g. the prominent glycoprotein at 72 kDa.

3.6 Discussion.

The lack of apparent binding of control serum with antigen after western blotting was predicted by its elimination after SDS treatment of the antigen sample prior to use for the immunodot binding assay. Solubilisation of proteins in the antigen sample by the SDS component of boiling mix possibly causes conformational changes which affect the appropriate epitopes. That the number of molecular species identified in S. lacrymans FPRL 12C standard mycelium with the antiserum to S. lacrymans FPRL 12C standard mycelium was less than the number identified by SDS-PAGE/silver stain was expected since not all proteins are necessarily immunogenic. These results are in accord with SDS-PAGE/silver staining and western blotting analysis of other Basidiomycetes, e.g. H. annosum (Galbraith, 1992).

Figure 17: The glycoprotein nature of the antigens of the standard mycelium of S. lacrymans FPRL 12C.



Track 7 represents low molecular weight standard proteins.
Tracks 8 and 12 represent high molecular weight standard proteins.
Tracks 1 - 6 represent S. lacrymans FPRL 12C standard mycelium stained for glycoproteins with the DIG Glycan detection kit.
Track 9 represents S. lacrymans FPRL 12C stained with India ink.
Tracks 10 and 11 represent immunostained S. lacrymans FPRL 12C.

o indicates antigens which are glycoproteins.
. indicates glycoprotein which is not an antigen.

The use of peroxidase labelled lectins to detect glycoproteins blotted onto nitrocellulose has been reported by several authors (Glass, Briggs & Hnilica, 1981; Kijimoto-Ochiai, Katagiri & Ochiai, 1985). The present study indicated that most of the antigens detected in S. lacrymans were glycoproteins. These results might have been predicted since polysaccharides usually comprise more than 75% (by weight) of the isolated fungal cell wall (Rosenberger, 1976) and glycoproteins and amorphous glucans predominate in the two outer walls of aged mycelium of some Basidiomycetes, e.g. S. commune, (Hunsley & Burnett, 1970). Additionally, other studies on purified, or partially purified, fungal antigenic components have identified the important role of carbohydrate (Hayashi et al., 1978; Schumacher et al., 1975) and glycoproteins (Gander, 1974; Graves et al., 1986; Hearn & M^{ac} Kenzie, 1979; 1980) in fungal antigenicity.

The dissimilarity observed between the staining patterns of glycoproteins identified by WGA and Con A was to be expected since each lectin has an affinity for different sugar residues. N-acetyl-β-D-glucosaminyl residues and β-D-glucosamine oligomers are the targets for WGA binding, whilst Con A has an affinity for α-D-mannosyl and α-D-glucosyl residues (Sigma) and the staining indicates significant amounts of these sugar residues in S. lacrymans. The variation shown in the frequency with which many of the antigens featured in the profile of S. lacrymans FPRL 12C standard mycelium mirrored similar variation between samples of standard mycelium of S. lacrymans FPRL 12C analysed by SDS-PAGE/silver staining and lectin staining. These results emphasised that this molecular variation is unlikely to be attributable to growth in uncontrolled conditions and indicates the existence of alternative sources of variation

within standard mycelium of S. lacrymans FPRL 12C which possibly relate to the genome.

The 2 major antigenic bands routinely detected in the standard preparation of S. lacrymans FPRL 12C (antigens 7 and 11) exhibited two specific features. Firstly, they do not correspond directly to any proteins detected in SDS-PAGE/silver stained profiles; this indicates either the high antigenicity of certain molecular components present at low levels in S. lacrymans FPRL 12C or a resistance of these components to the staining method of Blum, Beier & Gross (1987). Similar discrepancies have been reported in both overall banding patterns and the nature of bands within the patterns in SDS-PAGE/silver stained gels and western blots for Aspergillus fumigatus (Hearn et al., 1990), L. lepideus (Glancy, 1990) and C. puteana (M^{c}Dowell, 1992). Secondly, in contrast to the sharp banding patterns found on silver stained gels the major antigens appeared as very broad, indistinct bands. Two possible explanations can be proposed for this: either the major antigens have been degraded as a result of experimental procedures prior to antigen staining or the broad bands may represent extensively modified molecular species which are components of some aspect of mycelial structure or function. However, the occurrence of excessive proteolysis is not indicated since rigorous precautions to prevent protein degradation during the extraction of S. lacrymans antigens had little effect on the nature of the major antigens and India ink staining showed sharp banding patterns. Both features of the major antigenic bands are consistent with their previously established identity as highly antigenic glycoproteins; and the importance of glycoproteins as fungal antigens is well documented (Gander, 1974; Wycoff, Jellison & Ayers, 1987;

Hearn, Griffiths & Gorin, 1989). Antigens represented by defined bands are proteins which are possibly non-glycosylated, e.g. antigen 6.

That the antigenic bands are probably not representative of processes external to the standard mycelium, i.e. enzymes concerned with substrate degradation, is indicated by the use of washed mycelium as immunogen. These bands are probably representative of either intra-mycelial molecules, e.g. housekeeping gene products, or cell wall components. The production of diffuse bands after western blotting is not restricted to the profile of S. lacrymans since antisera that have been raised against similar preparations of other brown rot fungi, e.g. L. lepideus (Palfreyman et al., 1988a) and C. puteana (M^{c}Dowell, 1992) also produce diffuse bands. The production of diffuse antigenic bands may be related to the type of Basidiomycete under investigation since antisera similarly raised against two pathogenic white rot fungi, H. annosum (Galbraith, 1992) and Stereum hirsutum (M^{c}Cutcheon, 1989), produced well defined antigenic bands when used against standard mycelial extract.

One notable difference between the two fungi which are wood pathogens and those which decompose building timber relates to the ability of the environment to supply the requirements for growth. The environment of H. annosum and S. hirsutum has the potential to provide a constant supply of proteins from living ray tissue whereas there is a paucity of proteins in building timber (Deacon, 1984). The decomposers of building timber consequently must conserve proteins (Levi & Cowling, 1969) which S. lacrymans does by autolysis of older mycelium (Watkinson, 1975); a process which has not been reported for H. annosum and S. hirsutum. It is possible

that the diffuse antigens represented in the western blots of S. lacrymans FPRL 12C standard mycelium are representative of intra-mycelial processes associated with older mycelium concerned with autolysis and recycling of essential nutrients.

Similarity of the SDS-PAGE/silver stain, antigenic and glycoprotein profiles of most isolates to those of FPRL 12C would confirm identity as S. lacrymans; and the dissimilarity of all the profiles of BF-015B to those of FPRL 12C indicates incorrect identification as S. lacrymans. However, the extent of molecular difference which must be observed before two organisms can automatically be considered different species must be questioned because one putative S. lacrymans isolate, BF-050, had glycoprotein and antigenic profiles which were similar to FPRL 12C in spite of the marked differences between the respective SDS-PAGE/silver stain profiles. SDS-PAGE/silver staining is ideal for an identification aid since it is a highly discriminatory technique based upon analysis of a large number of proteins; but, since immunostaining detects fewer elements which are also possibly ubiquitous fungal components, similarity of antigenic profile could provide confirmation of identity which had been called into doubt by SDS-PAGE/silver staining.

CHAPTER 4. THE MOLECULAR ANALYSIS OF OTHER FUNGI.

4.1 General introduction.

The majority of isolates of S. lacrymans showed remarkable similarity to FPRL 12C when grown under identical conditions and analysed by SDS-PAGE/silver staining, western blotting and lectin staining but these analytical methods also indicated differences in two isolates. To determine whether inter-species differences in protein pattern were of the same order as the differences between isolates of one species a range of basidiomycete and non-basidiomycete fungi were analysed using the same techniques which were used for the range of S. lacrymans isolates.

The main objectives of the work reported in this chapter were

1. To use SDS-PAGE/silver staining to compare the profiles of standard mycelium of S. lacrymans FPRL 12C with profiles of standard mycelium from other fungal isolates.
2. To use lectin staining to extend the studies in 1.
3. To use the antiserum raised against standard mycelium of S. lacrymans FPRL 12C to compare the antigenic profiles of other Basidiomycetes with that of S. lacrymans FPRL 12C.

4.2 The protein profiles of other fungal organisms.

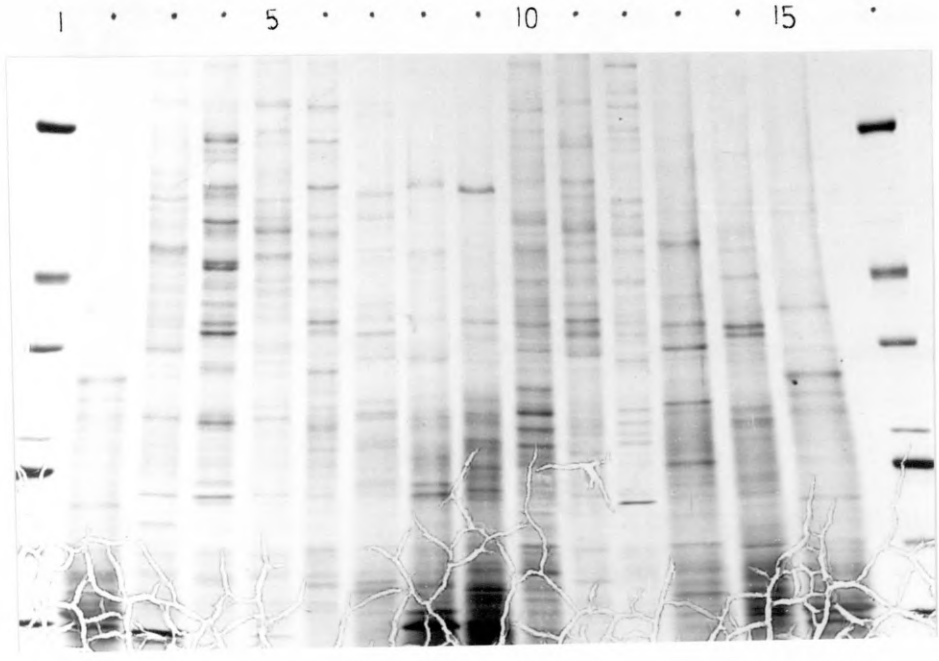
Other fungal organisms (Table 1) were analysed and compared with the standard S. lacrymans FPRL 12C preparation in order to determine if the SDS-PAGE/silver stained profile for standard mycelium of S. lacrymans

FPRL 12C was unique to the species. The fungi tested are all either known to rot building timbers or to affect wood by rotting and/or staining the felled timber prior to its use for building purposes. The results for one experiment are shown in Figure 18 and a diagrammatic representation of these bands is shown in Figure 19. The results indicated that none of the fungal extracts showed banding patterns similar to S. lacrymans FPRL 12C. Comparison of the data shown in Figures 18 and 19 with that shown in Figures 7 and 8 strongly indicates that, firstly, the profiles of each species are very different from that of S. lacrymans FPRL 12C; and, secondly, there are obvious differences between the profile of each species so that the overall pattern of bands is unique to each species.

4.3 The glycoprotein nature of other Basidiomycetes.

The sugar binding affinities of the lectins WGA and Con A were used to investigate the glycoprotein profile of standard mycelium from a range of Basidiomycetes in order to determine if the marked inter-species difference in protein profiles noted after SDS-PAGE/silver staining was supported by evidence from another technique. The profiles which resulted from WGA binding are shown in Figure 20 and indicate a diffuse staining of individual glycoproteins. There are marked differences in the glycoprotein banding pattern between the species of a variety of wood decay Basidiomycetes, results confirmed by Con A staining (data not shown).

Figure 18: SDS-PAGE/silver stain analysis of various wood inhabiting Basidiomycetes.



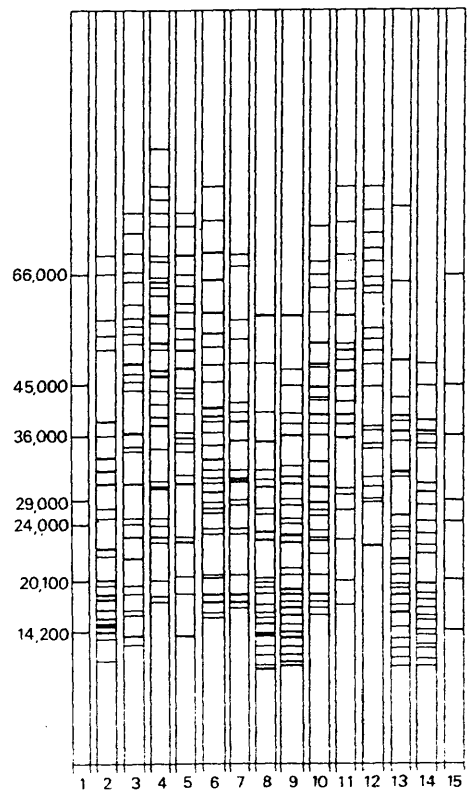
Tracks 1 and 16 represent low molecular weight standard proteins.

Tracks 2 and 15 represent standard extract of S. lacrymans FPRL 12C mycelium.

Tracks 3 - 14 represent the extracts of standard mycelium of the following fungi C. puteana (3); F. vaillantii (4); P. panuoides (5); A. xantha (6); S. pinastri (7); L.lepideus (8); G. sepiarium (9); P. gigantea (10); P. placenta (11); D. quercina (12); C. versicolor (13) and D. concentrica (14).

All samples were run at 3.12 mg/ml , with the exception of S. lacrymans, C. puteana, L. lepideus and G. sepiarium (all at 6.25 mg/ml).

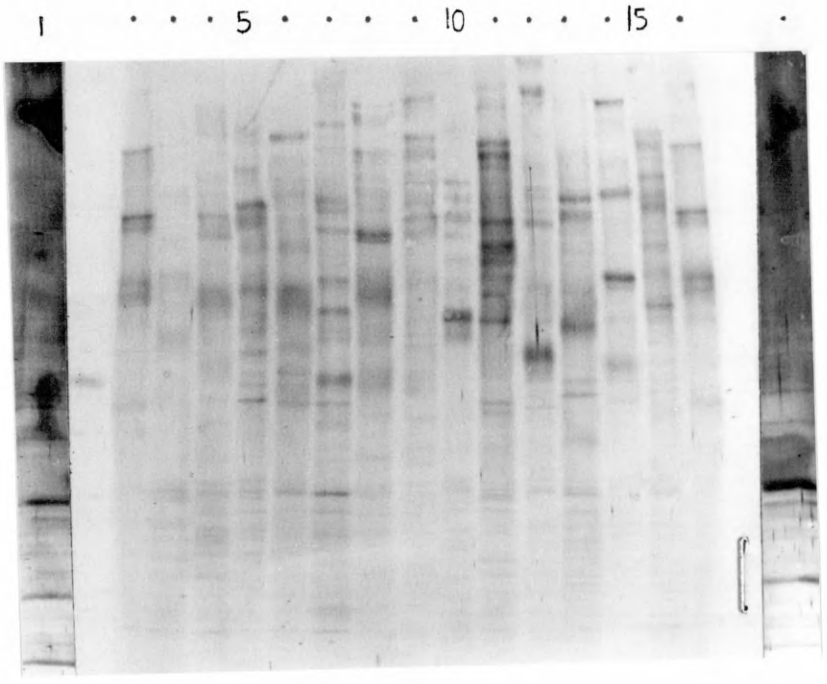
Figure 19: Diagrammatic representation of the SDS-PAGE/silver stained bands associated with S. lacrymans FPRL 12C and a range of other Basidiomycetes.



Tracks 1 and 15 represent low molecular weight standard proteins.

Tracks 2 - 14 represent S. lacrymans FPRL 12C (2); C. puteana (3); F. vaillantii (4); P. panuoides (5); A. xantha (6); S. pinastri (7); L. lepideus (8); G. sepiarium (9); P. gigantea (10); P. placenta (11); D. quercina (12); C. versicolor (13) and D. concentrica (14).

Figure 20: Analysis of the wheat germ agglutinin binding proteins of standard mycelium from a range of wood decay Basidiomycetes.



Tracks 1 and 17 represent low molecular weight standard proteins.

Tracks 2 - 16 represent standard mycelial preparations of the following species: S. lacrymans FPRL 12C (2, 16); D. concentrica (3); C. versicolor (4); D. quercina (5); P. placenta (6); P. gigantea (7); G. sepiarium (8); L. lepideus (9); S. pinastri (10); P. incrassata (11); A. xantha (12); P. panuoides (13); F. vaillantii (14) and C. puteana (15).

4.4 The antigenic profile of other Basidiomycetes.

The range of Basidiomycetes (Table 7) exhibited antigenic profiles distinct from S. lacrymans FPRL 12C and each other (Figure 21) following immunostaining. A degree of cross reactivity was noted with all the Basidiomycetes with the exception of D. concentrica (track 3). The two major diffuse antigens of S. lacrymans, antigens 7 and 11, were absent in all organisms except P. incrassata (track 11); and possibly P. panuoides (track 13) and, antigen 11 only, C. puteana (track 15). P. incrassata also had antigen 1, and possibly 5, in common with S. lacrymans FPRL 12C; P. panuoides possibly had antigen 2; whilst C. puteana had antigens 1, 3, 4 and 4.25 in common with S. lacrymans and possibly also had antigens 2, 5 and 6. Another experiment indicated that S. himantioides also had many antigens in common with S. lacrymans (data not shown). No other species possessed antigens found in the profile of S. lacrymans FPRL 12C.

Cross reactivity was particularly marked in some of the Basidiomycetes which, like S. lacrymans, cause brown rot of softwood in buildings, e.g. C. puteana, P. panuoides and P. incrassata. Figure 21 and Table 7 also indicate that these three species have profiles which resemble that of S. lacrymans in the diffuse character of the antigens detected. However, A. xantha and F. vaillantii (tracks 12 and 14 respectively), which also cause brown rot of constructional softwood, had no identifiable bands and their limited cross reactivity was indicated by smearing similar to that shown by C. versicolor, D. quercina and P. placenta (tracks 4 - 6 respectively).

Table 7: Types of decay (Cartwright & Findlay, 1958) caused by the Basidiomycetes investigated by western blotting and lectin staining.

Wood type.

1. Timber in buildings.

Fungi causing rot of softwoods	Fungi of minor importance
S. lacrymans	S. pinastri
C. puteana	
F. vaillantii	
P. panuoides	
A. xantha	

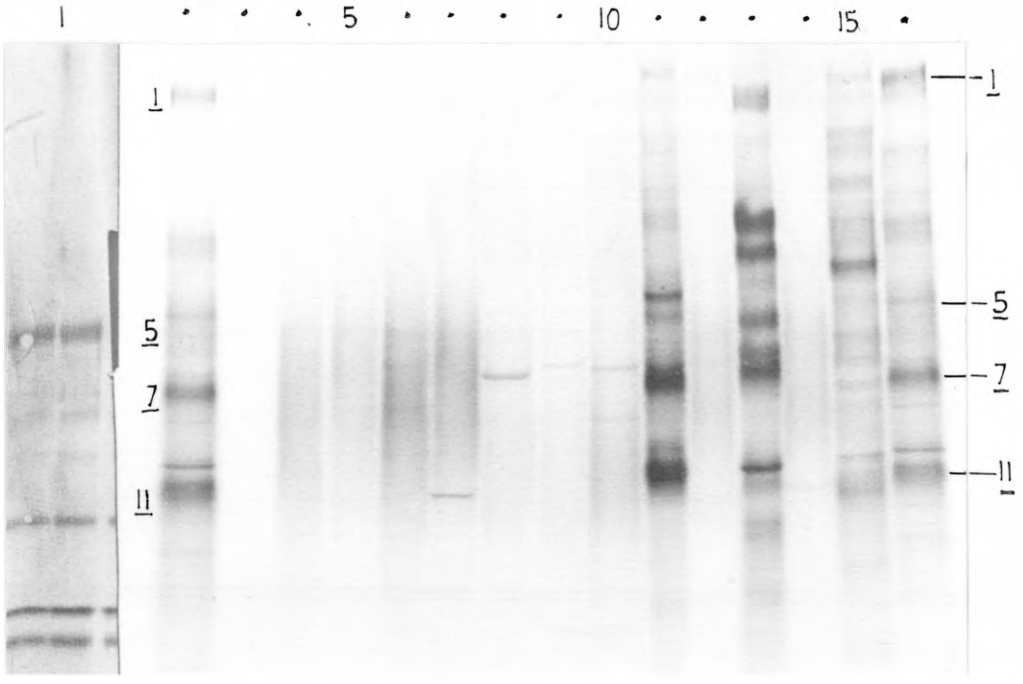
2. Felled timber and timber in service in the open.

Principal fungi on felled and worked softwoods	Fungi causing deterioration of felled and worked hardwoods
L. lepideus	D. quercina
G. sepiarium	C. versicolor
P. gigantea	
P. placenta	

3. Standing hardwood trees.

Fungi causing major decays of hardwoods
D. concentrica

Figure 21: Reaction of the antiserum to S. lacrymans FPRL 12C standard mycelium with the standard mycelium of a range of different Basidiomycetes.



Track 1 represents low molecular weight standard proteins.
Tracks 2 and 16 represent S. lacrymans FPRL 12C.
tracks 3 - 15 represent the following species: D. concentrica (3); C. versicolor (4); D. quercina (5); P. placenta (6); P. gigantea (7); G. sepiarium (8); L. lepideus (9); S. pinastri (10); P. incrassata (11); A. xantha (12); P. panuoides (13); F. vaillantii (14) and C. puteana (15).

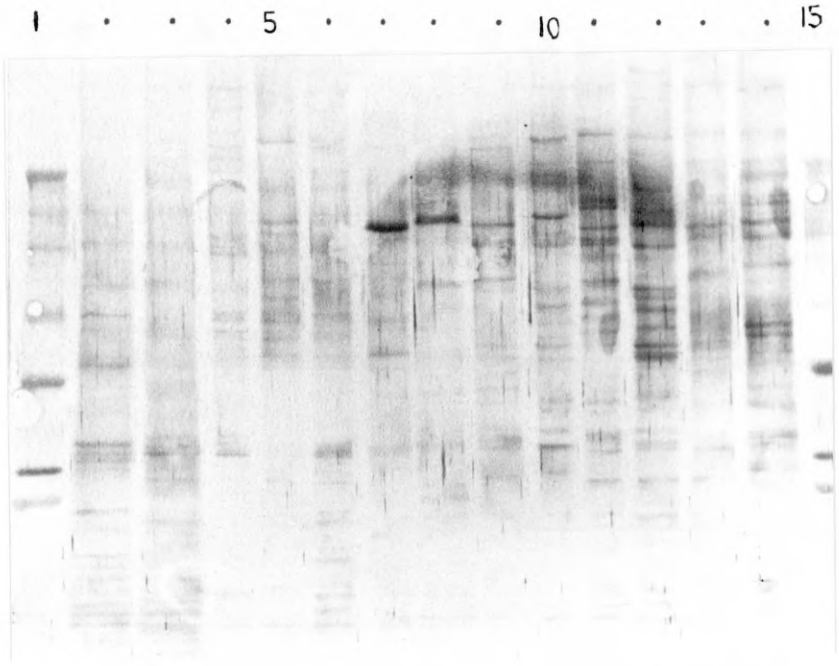
Antigens 1, 5, 7 and 11 are marked thus 1, 5, 7, 11.

Profiles containing only one defined cross reacting antigen were shown by the organisms which are are responsible for decay of softwood in the open, e.g. S. pinastri , P. gigantea, G. sepiarium and L. lepideus (tracks 7 - 10 respectively). The diffuseness of some antigen bands is similar to the bands obtained after lectin staining (Figure 20) and these are in contrast to the sharp bands displayed after SDS-PAGE/silver staining and India ink staining (Figure 22).

4.5 Discussion.

SDS-PAGE/silver staining, western blotting and lectin staining have all indicated that other basidiomycete species had profiles which were distinctly different from the profiles which had been observed within the majority of the S. lacrymans isolates; thus indicating that the inter-species difference between protein profiles is greater than differences observed between profiles within the species S. lacrymans. This conclusion supports previous experimental results, in this thesis and by Schmidt & Kebernik (1989) which indicated that S. lacrymans isolate BF-015B was not a member of the species S. lacrymans. Analysis of SDS-PAGE/silver stain protein patterns and western blots has previously been used for differentiation of Basidiomycetes such as C. puteana (McDowell, 1992) and H. annosum (Galbraith, 1992) from other fungal isolates and the results of present studies indicate that these molecular techniques have the potential to be used similarly on S. lacrymans.

Figure 22: India ink stained protein profiles of some Basidiomycetes after electroblotting.



Tracks 1 and 15 represent low molecular weight standard proteins.

Tracks 2 - 14 represent tracks of the following Basidiomycetes: D. concentrica (2); C. versicolor (3); D. quercina (4); P. placenta (5); P. gigantea (6); G. sepiarium (7); L. lepideus (8); S. pinastri (9); A. xantha (10); P. panuoides (11); F. vaillantii (12); C. puteana (13) and S. lacrymans FPRL 12C (14).

There was a wide variation in the cross reactivity of different Basidiomycetes with the test antiserum but the results indicated that the degree of cross reactivity is not related to the nature of the organism as a brown or white rot fungus, e.g. two brown rot organisms, A. xantha and F. vaillantii, showed as little cross reactivity as C. versicolor and D. concentrica, two white rot organisms. That the degree of cross reactivity is variable could also be attributable to the wood type decayed by an organism. In support of this, D. concentrica, a pathogen decaying hardwood trees, showed no cross reactivity and it was observed that the greatest degree of cross reactivity was found in some of those species which cause brown rot of softwood in buildings, e.g. C. puteana, P. panuoides and P. incrassata. However, A. xantha and F. vaillantii also decay softwood in buildings and their profiles are more similar to those of organisms which decay felled timber in the open. It is unlikely, therefore, that the degree of cross reactivity to the test antiserum is directly related to the environment of the organism and is more probably associated with the presence of ubiquitous fungal proteins in standard mycelium.

Investigation of antigenic profiles possibly indicates that the more closely related taxonomically the organisms are the more components their profiles have in common. This is supported, firstly, by the fact that most organisms in families other than the Coniophoraceae were found to have no common antigens with S. lacrymans, e.g. C. versicolor, L. lepideus and A. xantha; and, secondly, by the presence of common antigenic species in the profiles of C. puteana, P. incrassata, S. lacrymans and S. himantioides. These all belong to genera within the Coniophoraceae (Rayner & Boddy, 1988), C. puteana is the type species of Coniophora; and P.

incrassata, assigned by Donk to the genus Serpula in 1948 (Decock & Hennebert, personal communication), has more recently been adopted as the type species of Meruliporia, a genus which is included in the Coniophoraceae (Pegler, personal communication). Since isolate BF-015B has an antigenic profile which apparently shows more similarities to that of S. lacrymans FPRL 12C than any other Basidiomycete investigated it might be that BF-015B belongs to a species which is very closely related to S. lacrymans.

However, whilst similarity in antigenic profiles might be useful for confirmation of organism identity it is doubtful if such profiles could be used reliably as indicators of taxonomic affinities. This is indicated, firstly, by the antigenic profile of P. panuoides, which has antigenic bands in common with S. lacrymans but which belongs to the Agaricaceae, a family in the Agaricales, i.e. a different order to the Aphyllophorales which contains the Coniophoraceae (Ainsworth, 1963). Such common antigenic bands are probably representative of ubiquitous fungal antigens. Secondly, the absence of common antigens in the profiles of S. pinastri and S. lacrymans is at odds with their present taxonomic relationship since, despite a recent reassignation to the genus Leucogyrophana (L. pinastri (Fr.) Ginns & Weres. (Pegler, personal communication)), S. (L.) pinastri remains within the Coniophoraceae (Rayner & Boddy, 1988). It could be, however, that absence of common antigens in the profile of a putative member of a family is indicative of incorrect taxonomic assignation. S. tignicola has also been reassigned to the genus Leucogyrophana (L. pulverulenta (Sow:Fr) Ginns (Pegler, personal communication) and it would be of interest to similarly investigate the nature of its antigenic profile.

CHAPTER 5. NUMERICAL ANALYSIS OF MOLECULAR PROFILES.

5.1 General introduction.

The molecular methods which have been used in this study have shown that SDS-PAGE/silver staining and western blotting can, firstly, allow comparisons to be made between *S. lacrymans* and other fungal species and, secondly, enable organisms to be identified. However, to develop these techniques as routine comparative systems it is necessary to collate and assess information on the similarities and differences of the isolates under investigation.

The main objectives of the work reported in this chapter were

1. A comparison of the feasibility of using two possible numerical methods to assess the similarities and differences in fungal organisms detected by SDS-PAGE/silver staining and western blotting techniques.
2. To numerically compare other fungal organisms to *S. lacrymans* FPRL 12C.
3. To evaluate the use of molecular methods as tools to aid in fungal identification.

5.2 Analysis of profiles.

5.2.1 Laser densitometry.

A possible method of numerical analysis would be based on interpretation of gel patterns by laser densitometry linked to computer analysis. However, two problems were associated with this method of analysis in the present study. Firstly, the absence of appropriate equipment for

analysis of stained Immobilon and, secondly, the absence of computer software to handle data generated by scanning of SDS-PAGE/silver stained gels.

5.2.2 Visual analysis.

An alternative method of analysis involved the use of the map of the SDS-PAGE/silver stained patterns for each of the isolates tested (Figure 8) for the manual calculation of the protein percentage similarities for isolates of S. lacrymans FPRL 12C (Table 8). The protein similarity index for S. lacrymans isolates ranged from 46.1 - 100 and confirmed that although the majority of isolates showed remarkable similarity to FPRL 12C when grown in identical conditions two isolates were appreciably different, i.e. most isolates of S. lacrymans had protein percentage similarities to FPRL 12C of >98%, but those of BF-050 and BF-015B were 68.4% and 46.1% respectively. The range of the antigenic similarity index for S. lacrymans isolates (data not shown) was from 50, isolate BF-015B, to 100, all other isolates.

The protein similarity index for a range of fungal species is shown in Table 9 and ranges from 0.0 (D. quercina) - 43.5 (S. himantioides). Comparison between Table 8 and Table 9 indicates, firstly, that the protein similarity indices for isolates within S. lacrymans and for other fungal species have mutually exclusive ranges and, secondly, that isolate BF-015B is the only S. lacrymans isolate whose percentage similarity is similar to non-S. lacrymans species. The antigenic similarity index for non-S. lacrymans isolates ranged from 0 - 58 and confirmed visual impressions (Figure 21) since, with

Table 8: Protein similarity index of S. lacrymans isolates in reference to isolate FPRL 12C.

Isolate number	Percentage similarity	Number of observations
CMI 152233	100	4
BF-03A	100	3
BF-07B	100	4
BF-017B	100	4
BF-023	100	3
BF-025	100	3
BF-044	100	2
BF-046	100	2
BF-049	100	2
BF-072	100	4
MAD 90876R	100	2
BAM 315	100	2
BF-01	99	5
BF-018A	98	4
BF-050	68.4	6
BF-015B	46.1	7

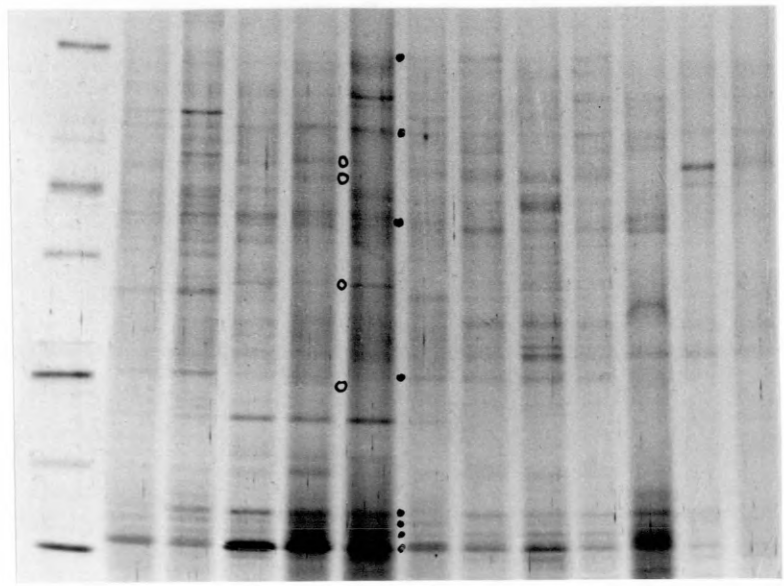
Table 9: Protein similarity index of non-*S. lacrymans* species in reference to *S. lacrymans* FPRL 12C.

Species	Percentage similarity	Number of observations
S. himantioides	43.5	3
S. pinastri	36.7	4
M. tremellosus	34.8	2
G. trabeum	34.3	2
S. tignicola	29.1	2
F. vaillantii	28.1	7
S. sanguinolentum	26.5	2
D. concentrica	24.0	3
P. incrassata	24.0	3
S. commune	21.7	2
H. annosum	21.5	2
C. resinae	21.2	1
L. lepideus	20.1	4
P. panuoides	19.0	4
A. xantha	18.4	4
G. sepiarium	17.4	4
C. puteana	17.3	6
C. versicolor	16.5	4
P. placenta	15.6	3
P. ostreatus	15.2	2
P. gigantea	10.7	2
P. variotti	7.9	2
D. quercina	0.0	2

the exception of S. himantioides, C. puteana, P. incrassata and P. panuoides (similarities of 58%, 47%, 30.8% and 21.5% respectively), all other species had no antigenic similarity to S. lacrymans FPRL 12C.

Mycelial, strand and basidiocarp material of S. lacrymans was collected from 2 local sites, DIT-101 and DIT-102, and analysed either directly (mycelium, basidiocarp and strand) or isolated (mycelium) and subsequently used for preparation of standard mycelium. The results of SDS-PAGE/silver stain analysis are shown in Figure 23 and indicate that only standard mycelium grown from recent isolations of S. lacrymans DIT-101 (Figure 23, track 3) and DIT-102 (Figure 23, track 4) showed a molecular profile which was very similar to that of FPRL 12C. Samples analysed without isolation showed bands in common with FPRL 12C and also some new bands, e.g. compare mycelium (Figure 23, tracks 4 - 6) with standard mycelium of S. lacrymans FPRL 12C (track 7). The protein similarity index of all samples investigated is shown in Table 10. The visual similarity of the protein pattern of isolates DIT-101 and DIT-102 to isolate FPRL 12C is confirmed by their protein percentage similarity of 100; whereas the visual dissimilarity of non-isolated material to isolate FPRL 12C is confirmed by the protein percentage similarities of 38.2 - 41.9% (mycelium, Figure 23, tracks 4 - 6) and 40% (basidiocarp). Since <20 protein bands were identified in strands and no proteins were detected in spores it was not possible to determine the percentage similarity to FPRL 12C of either of these morphological forms.

Figure 23: The constancy of the SDS-PAGE/silver stained profile of standard mycelium of S. lacrymans FPRL 12C.



Track 1 represents low molecular weight standard proteins.
Tracks 2 and 7 represent standard mycelium of S. lacrymans FPRL 12C.
Track 3 represents S. lacrymans DIT-101 mycelial isolate.
Track 4 represents S. lacrymans DIT-102 mycelial isolate.
Tracks 5 and 6 represent mycelium scraped from stonework at DIT-101 (samples 1 - 3).
Tracks 8 - 10 represent basidiocarp from DIT-101 (samples 1 -3).
Track 11 represents basidiocarp from DIT-102.
Tracks 12 and 13 represent strand from DIT-101 and DIT-102 respectively.

. represents bands in common with FPRL 12C.
o represents bands not found in FPRL 12C.

Table 10: The protein similarity index of different preparations of *S. lacrymans* with reference to *S. lacrymans* FPRL 12C.

Sample	Site	Percentage similarity
Mycelial isolate	DIT-101	100
Mycelial isolate	DIT-102	100
Field mycelium (sample 1)	DIT-102	38.2
Field mycelium (sample 2)	DIT-102	40.6
Field mycelium (sample 3)	DIT-102	41.9
Basidiocarp (sample 1)	DIT-101	40.0
Basidiocarp (sample 2)	DIT-101	40.0
Basidiocarp (sample 3)	DIT-101	40.0
Basidiocarp	DIT-102	36.0
Strand	DIT-101	*
Strand	DIT-102	*
Spores	DIT-101	-
Spores	DIT-102	-

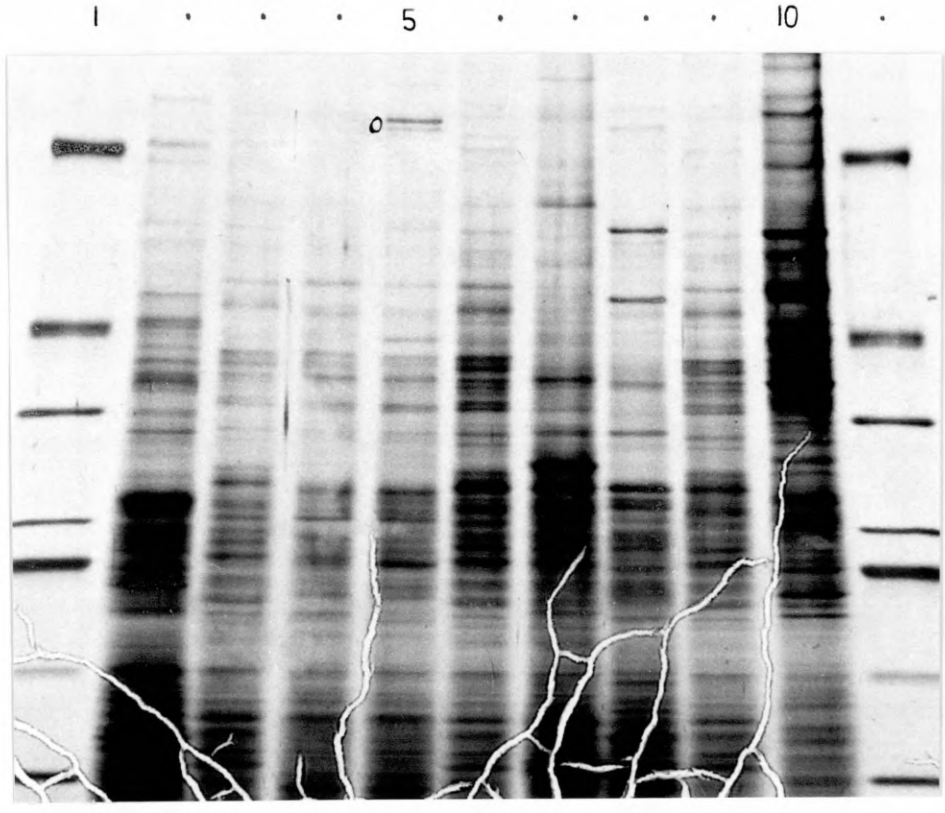
* indicates less than 20 bands on track.
\- indicates no proteins detected.

5.3 The use of molecular methods for fungal identification.

Molecular analysis of standard mycelium has potential for the identification of organisms, so the identity of putative S. lacrymans isolates BF-050 and BF-015B was further investigated. A comparison of an analysis by SDS-PAGE/silver stain of BF-050 and BF-015B with other relevant organisms all currently or formerly members of the genus Merulius (Cartwright & Findlay, 1958; Rune & Koch, 1992), viz S. lacrymans FPRL 12C (M. lacrymans Wulf : Fr), S. himantioides (M. himantioides Fr : Fr), S. pinastri (M. pinastri (Fr : Fr) Burt), S. tignicola (M. tignicola Harmsen), M. tremellosus and P. incrassata (M. spissus Berk), is shown in Figure 24. Isolates BF-015B (track 5) and BF-050 (track 6) illustrate the differences previously observed (Figures 7 and 8) between themselves and S. lacrymans (track 7) and demonstrate that the profile of BF-015B is more similar to that of S. himantioides (track 4) than to that of S. lacrymans. Some differences can be observed between BF-015B and S. himantioides, e.g. the marked pair of proteins at 72 and 74 kDa in BF-015B. Table 11 shows the protein similarity indices of BF-015B (Table 11A) and BF-050 (Table 11B) which confirms that BF-015B is as similar to S. himantioides (68.6%) as isolate BF-050 is to S. lacrymans FPRL 12C and that each is not more similar to any other species in the comparison.

The similarities between organisms was also tested by western blotting (Figure 25). There was greater visual similarity between the profile of isolate BF-015B (track 11) and S. himantioides (track 10) than either S. lacrymans (track 12) or any other species and this was

Figure 24: Analysis by SDS-PAGE/silver staining of S. lacrymans isolates BF-050 and BF-015B.



Tracks 1 and 11 represent molecular weight standard proteins.

Tracks 3, 6 and 9 represent extracts of isolate BF-050.

The following tracks represent M. tremellosus (2); S.himantioides (4); isolate BF-015B (5); S. lacrymans FPRL 12C (7); S. pinastri (8); S. tignicola (10).

o represents proteins at 72 and 74 kDa.

Table 11: The protein similarity indices of BF-050 and BF-015B.

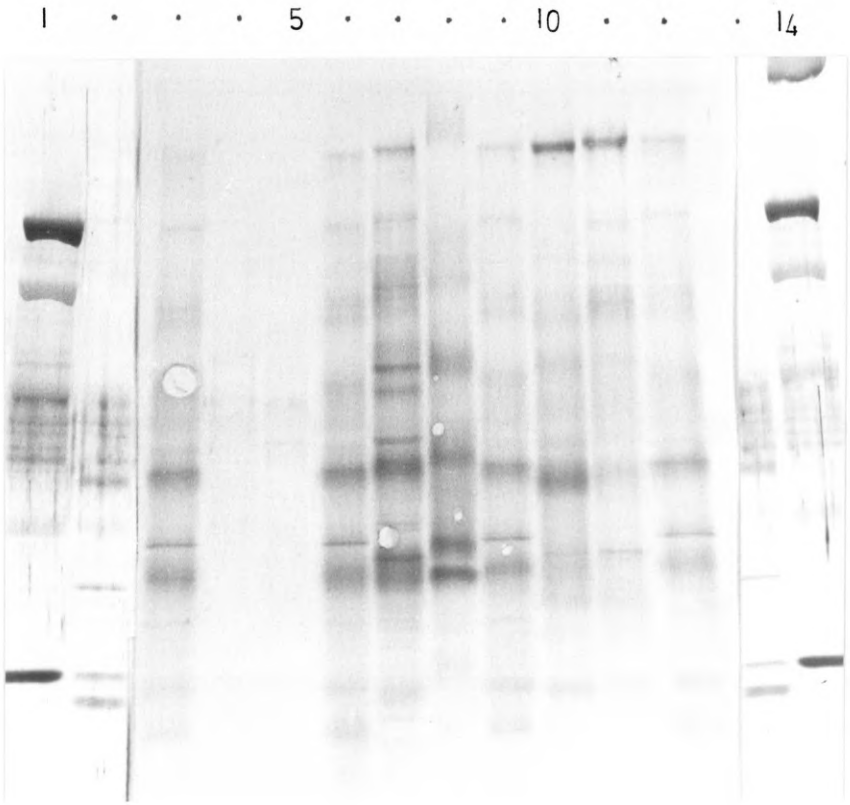
Table 11A: Protein percentage similarity of BF-015B to different fungal isolates.

Reference species	Percentage similarity of BF-015B	Number of observations
S. himantioides	68.6	4
S. lacrymans FPRL 12C	46.1	7
isolate BF-050	44.7	3
S. tignicola	40.0	3
M. tremellosus	28.6	2
S. pinastri	20.0	2

Table 11B: Protein percentage similarity of BF-050 to different fungal isolates.

Reference species	Percentage similarity of BF-050	Number of observations
S. lacrymans FPRL 12C	68.4	6
S. himantioides	47.1	2
isolate BF-015B	43.4	3
S. tignicola	38.0	1
M. tremellosus	29.4	1
S. pinastri	26.3	2

Figure 25: Western blot analysis of isolate BF-015B and some species formerly within the genus Merulius (Cartwright & Findlay, 1958).



Tracks 1 and 14 represent high molecular weight standard proteins.

Tracks 2 and 13 represent low molecular weight standard proteins.

Tracks 3, 6, 9 and 12 represent S. lacrymans FPRL 12C.

The following tracks represent M. tremellosus (4); S. pinastri (5); P. incrassata (7); S. tignicola (8); S. himantioides (10); isolate BF-015B (11).

confirmed by numerical analysis. The antigenic percentage similarities of BF-015B to S. himantioides (2 analyses), S. lacrymans FPRL 12C and P. incrassata were 73, 50 and 40 respectively. There was no antigenic similarity between BF-015 and either S. tignicola, M. tremellosus or S. pinastri. Previous work had established that the antigenic profile of isolate BF-050 was visually identical to FPRL 12C (Figure 13) and, therefore, had an antigenic percentage similarity to FPRL 12C of 100.

5.4 Discussion

S. lacrymans isolates gave SDS-PAGE/silver stain and antigenic patterns which were visually more similar to each other than the patterns produced by different Basidiomycete species but problems were posed in the numerical analysis of such patterns. However, the construction of protein and antigenic similarity indices enabled objective comparison of these results and, whilst the quantitative nature of the similarity indices may be susceptible to criticism because of possible subjectivity, they reflected the preliminary visual impressions. This has now been confirmed by the construction of similarity indices for wood decay Basidiomycetes isolated from the historic ships Discovery and Unicorn (M^{c}Dowell & Palfreyman, 1992) and for certain forest pathogens (Galbraith, 1992).

However, two aspects of a similarity index should be stressed. Firstly, whilst comparative assessments between several tracks is facilitated by the use of a

similarity index, initial comparison of two gel patterns by visual observation is likely to provide an assessment of the similarity of pattern that is as accurate as either the protein or antigenic percentage similarity of the test isolate. Secondly, since a similarity index quantifies differences and similarities between test tracks and a reference track it could not easily be used for taxonomic purposes since the aims of numerical taxonomy are to clarify degrees of relatedness of a large number of similar organisms according to their characters and to group them by numerical methods into taxonomic units (Sneath & Sokal, 1985). SDS-PAGE/silver staining has been extensively used for taxonomic purposes in the fungi, e.g. Maas et al., (1990); Chen, Hoy & Schneider (1991), but it is necessary to use computer analysis of electrophoretic protein patterns (Jackman, Feltham & Sneath, 1983; Kersters, 1985) to produce the shadowed matrices or dendrograms (Kersters, 1985) which indicate possible taxonomic relationships.

However, the protein and antigen similarity indices have largely confirmed visual impressions that the difference in banding pattern observed within a species is not of the same order as that observed between species. Therefore, similarity indices could offer highly precise and relatively simple means of identification of new isolates of S. lacrymans and differentiation of non-S. lacrymans species. This method of identification could be exploited by either those who are unskilled in the traditional methods for the identification of fungi or experts seeking confirmation of identity. This method of identification has been successfully applied to the Basidiomycetes C. puteana and L. sulphureus (M^{c}Dowell,

1992); H. annosum (Galbraith, 1992) and G. trabeum (Hainey, personal communication) and was used in this study for the identification of S. lacrymans isolates DIT-101 and DIT-102. If protein profiles allied with similarity indices are to be used for identification of S. lacrymans then it is important that different species which are likely to be found in a similar environment have profiles whose differences can be quantified. The similarity indices constructed for S. lacrymans and non-S. lacrymans species indicated that this criterion can be satisfied. The importance of standardisation of culture methods prior to the use of percentage similarities for identification of S. lacrymans was emphasised by the protein similarity index for isolated and non-isolated field material. This indicated that only isolated mycelium could be identified with certainty as S. lacrymans and underlines the present use of isolated mycelium for identification purposes. In spite of standardised culture methods there are some physiological features, e.g. growth rates, which cannot be equalised; but previous work has indicated that banding patterns obtained by analysis of S. lacrymans did not relate to growth rates.

Identification of S. lacrymans is important because different remedial processes are required to counter the effects of this organism compared to other wood decay Basidiomycetes (Bravery et al, 1987). Currently field identification by experienced professionals working in remedial timber treatment depends upon initial visual examination of suspect timber. Personal experience combined with use of texts, e.g. Cartwright & Findlay (1958) or Bravery et al (1987), are subsequently the

normal methods for distinguishing between the wood decay Basidiomycetes. Diagnosis can be confirmed by isolation studies and use of specific keys, e.g Nobles (1965) or Stalpers (1978), which relate information on a range of physiological and morphological characteristics of an unknown isolate. However, identification of S. lacrymans by less experienced personnel is not straightforward, especially in the absence of fruit bodies, and occasionally disputes arise, with major financial implications, around the identification of particular outbreaks of decay fungi.

Identification of unknown isolates as S. lacrymans usually poses few problems to mycologists. The fruit body and fresh mycelium are easily distinguished from those of other Basidiomycetes, with the possible exception of S. himantioides, whilst in laboratory culture the organism shows a number of specific characteristics, e.g. its unusual heat sensitivity and low temperature growth optimum. Mycologists use a range of methods to identify Basidiomycetes, e.g. the presence or absence of clamp connections in the mycelium; analysis of the growth on selective media (Hunt & Cobb, 1971); and the use of complex keys, but all are time consuming procedures, especially the latter which can take up to six weeks to get organism identity (Nobles, 1965), and none of them are infallible.

Whilst the protein and antigenic similarity indices have confirmed that the majority of S. lacrymans isolates are identical to the reference isolate they have also quantified the observed differences of two putative isolates of S. lacrymans, BF-050 and BF-015B. This would

add credence to doubts previously raised about the identity of these isolates after visual observations on their protein profiles and it could be that the use of different reference species to construct similarity indices could aid identification of these isolates.

It is possible that molecular profiles are most similar amongst taxonomically closely related organisms and identification of the two exceptional isolates of S. lacrymans involved comparison of their molecular profiles with a range of species which, like S. lacrymans, have been classified in the genus Merulius. Interestingly, the protein and antigenic percentage similarities, 68.6 and 73 respectively, of isolate BF-015B were more similar to S. himantioides than to any other species analysed. This is the only other member of the present genus Serpula and is said to represent S. lacrymans outwith buildings (Cartwright & Findlay, 1958). However, since it is also found within buildings and its mycelial form has similarities to S. lacrymans, mistake in identification is possible. Additional support for this identification is provided by the protein percentage similarities to S. lacrymans FPRL 12C of S. himantioides and BF-015B which, at 43.5 and 46.1 respectively, are very similar. It is probable that BF-015B is confirmed as an isolate of S. himantioides by the use of similarity indices.

The antigenic percentage similarity of 100 for BF-050 would indicate that this isolate had been correctly identified as S. lacrymans were it not for the anomaly of its relatively low protein percentage similarity to S. lacrymans FPRL 12C. However, since the similarity

indices of BF-015B probably confirm identity as S. himantioides, the indices of BF-050 must similarly confirm identity as S. lacrymans. These conclusions support those of Schmidt & Kebernik (1989) who indicated that isolate BF-050 was an isolate of S. lacrymans but BF-015B was not; it was subsequently suggested on the basis of growth studies that BF-015B was an isolate of the closely related S. himantioides (Schmidt, personal communication). Since the protein percentage similarity of BF-050 is markedly outwith the range of the protein similarity index for non-S. lacrymans isolates this is compatible with the identification of the organism as S. lacrymans. Since it is acknowledged that there are subtle differences between individuals at the species and sub-species level (Foster, 1949) the protein percentage similarity of isolate BF-050 was assumed to represent variations within the species.

The difference between the protein percentage similarity of S. lacrymans BF-050 and that of other isolates of S. lacrymans analysed suggest that there are other, non-molecular, features of the organism which would differentiate it from the other isolates. However, no obvious differences in morphology between BF-050 and the other isolates have been identified to date and Cymorek & Hegarty (1986), who analysed a number of characteristics of 25 isolates of S. lacrymans, reported no significantly different features of BF-050 in terms of growth rate or decay of timber. An alternative cause might be the geographical site of origin of an isolate. However, whilst BF-050 is an Australian isolate, so also are BF-046 and BF-049. It is interesting to speculate that BF-050 may represent the first stages of a

speciation process. If this is the case then it must be concluded that this process affects whole cell proteins of the organism before either the antigenic or glycoprotein components. The discrepancy between the profiles of BF-015B and S. himantioides indicated by the antigenic percentage similarity of BF-015B might suggest that the process of speciation is more fully developed in isolate BF-015B than in BF-050.

Work in this chapter has indicated that the construction of similarity indices can quantify differences in molecular profiles and enable isolated mycelium to be identified as S. lacrymans. However, it has yet to be established conclusively whether non-isolated material, either different morphological forms or within wood, can be identified as S. lacrymans in this way. The present work has indicated that standard mycelium is not appropriate as a reference sample to establish percentage similarity indices which enable non-isolated S. lacrymans to be identified. Criticism which could be directed against the present use of similarity indices for identification would centre round the subjective nature of assessment of the bands in the protein or antigenic profile. However, scanning equipment linked to appropriate computer software could remove this subjectivity and would allow similarity indices to be constructed which would be highly reliable indicators of identity.

CHAPTER 6. THE EFFECT OF CULTURAL FACTORS ON THE MOLECULAR PROFILE OF *S. lacrymans* FPRL 12C.

6.1 General introduction.

Studies reported in chapters 3 - 5 have analysed S. lacrymans mycelium grown in standardised conditions which resulted in reproducible molecular profiles. In the natural environment conditions are anything but standardised, and a small study in chapter 5 had indicated that the protein profile of the non-isolated organism could be expected to vary. However, no assessment had yet been made about the effect of environmental factors on the profile of S. lacrymans FPRL 12C. It is possible for S. lacrymans to be affected in the laboratory by subculture, temperature, other organisms and contents of medium. Studies were initiated into the effects of these parameters on the molecular profiles of S. lacrymans in order to identify any changes in molecular nature due to different cultural conditions prior to investigation of profiles resulting from growth in an uncontrolled environment.

The main objective of work in this chapter was to assess the effect of lethal factors and variations in the established method of culture upon the SDS-PAGE/silver stain and/or antigenic profiles of S. lacrymans FPRL 12C mycelium. The factors which were investigated were

1. Basic parameters of culture:
    a. Growth on solid medium.
    b. Washing stages prior to freeze drying.
    c. The age of the culture used for growth of standard mycelium.
    d. The period between inoculation and harvest of mycelium.
2. The total nitrogen (TN) content of the medium.

3. The harvest of different regions of mycelium represented by young and aged mycelium.
4. The effect of exposure to lethal conditions.
   a. A temperature of 40°C.
   b. 3 species of Trichoderma.

6.2 Variation in the basic parameters of culture.

SDS-PAGE/silver staining and western blotting were used to determine whether the protein banding pattern or the major antigens of S. lacrymans FPRL 12C were affected by the type of growth medium, the washing stages after harvest of mycelium, the age of culture inoculated for growth of mycelium, or the time of harvest of mycelium. The results of two analyses are shown in Table 12 (protein similarity index) and Figure 26 (western blot) and indicate that all harvests had essentially identical protein and antigenic profiles, e.g. compare Figure 26, tracks 2 and 3 or 3 and 14. The major proteins and antigens of S. lacrymans FPRL 12C standard and agar grown mycelium harvested after different times and from different ages of subculture were similar; thus indicating that neither subculture, age of culture, type of medium nor the washing stages had a marked effect on the proteins and antigens detected by the two techniques.

6.3 The effect of TN content of the growth medium.

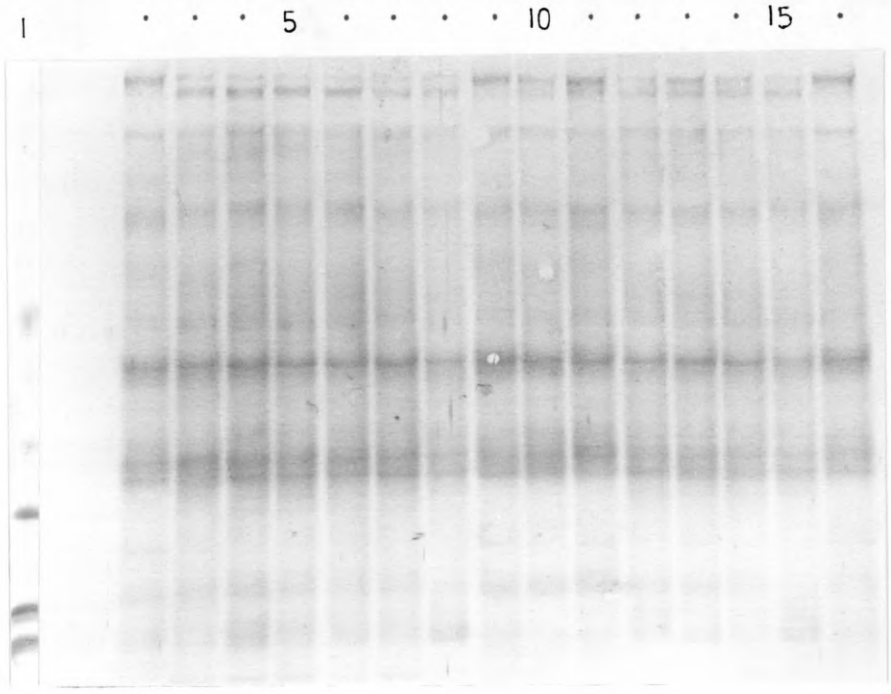
Visual observations made whilst S. lacrymans was under culture indicated that the amount of TN in the medium

Table 12: Protein similarity index of S. lacrymans FPRL 12C after variation in basic cultural parameters.

Subculture number	Age at use (months)	Uplift (days)	Percentage similarity	
			Standard mycelium	Agar grown mycelium
1	0	3	98	99
1	0	5	99	98 (2)
1	0	7	100 (2)	99
1	0	10	99	100
2	2	10	98 (2)	99
3	6	10	98 (2)	100 (2)

All analyses were made in triplicate unless indicated otherwise by the figure in parenthesis, ( ).

Figure 26: The effect of the variation of basic cultural parameters on the antigenic profile of S. lacrymans FPRL 12C.



Track 1 represents low molecular weight standard proteins.

Tracks 2, 9 and 16 represent standard mycelium of S. lacrymans FPRL 12C.

Tracks 3 - 8 represent agar grown mycelium and tracks 10 - 15 represent material harvested from liquid culture.

Subculture at 0 weeks is represented by tracks 5 - 8 and 12 - 15; tracks harvested at 10, 7, 5 and 3 days respectively.

Subculture at 2 months is represented by tracks 4 and 11; and subculture at 6 months is represented by tracks 3 and 10.

had a marked effect upon the appearance of the resultant growth from an inoculum (Figure 27). Hyphae formed a denser mycelium in media containing higher amounts of TN than those growing in media with a lower concentration of TN, e.g. compare plates 1 or 2 with 4 or 5. Additionally, there was a marked visual difference between mycelium grown on 5% MEB containing 2.36% TN (plate 1) and that grown on medium containing 2.36% TN derived from mycological peptone only (plate 2); equally noticeable was the paucity of growth from an inoculum in medium containing no TN (plate 5).

SDS-PAGE/silver staining (Figure 28) and western blotting (Figure 29) indicated that no antigens or proteins were detected in the media within which S. lacrymans had been cultured, e.g. Figure 29, tracks 3 - 7. Few proteins were detected by SDS-PAGE/silver staining in mycelium which had been cultured in 0% TN (Figure 28, tracks 11 and 12) and the number of proteins appeared to be directly related to the TN content of the medium. Both analyses indicate that the overall banding pattern does not change markedly provided that the medium contains a source of TN (Figure 29, tracks 8 - 16). However, SDS-PAGE/silver stain analysis indicates that some major proteins change their nature as the TN content of the medium decreases, e.g. 35 kDa and 15.5 kDa which cease to be major proteins below 2.36% TN. Some major proteins also appear, e.g. 22.5 kDa which appears in mycelium grown in media prepared from mycological peptone and which contain 2.36% TN and 0.127% TN (tracks 5 - 8). The only notable difference shown between antigenic profiles is antigen 19 (12 kDa)

Figure 27: The morphological appearance of mycelium of *S. lacrymans* FPRL 12C after 7 days culture in liquid media containing differing amounts of total nitrogen (TN).

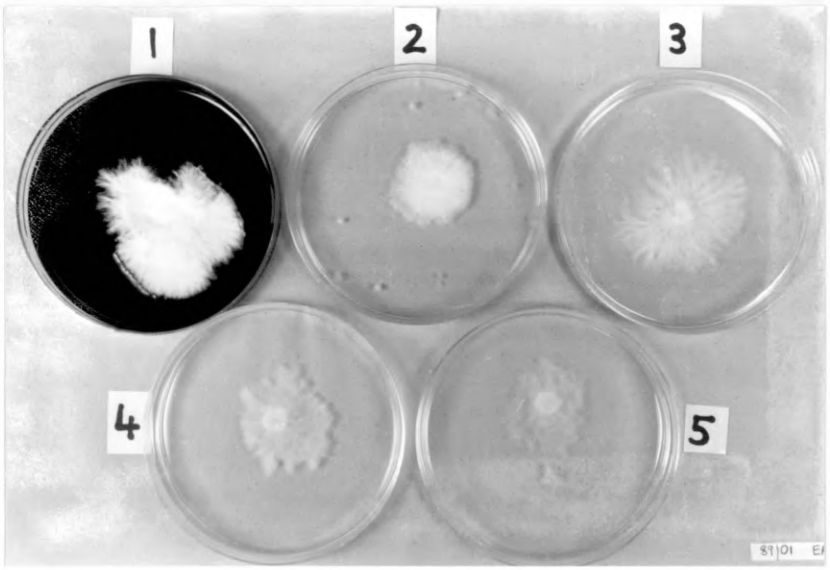
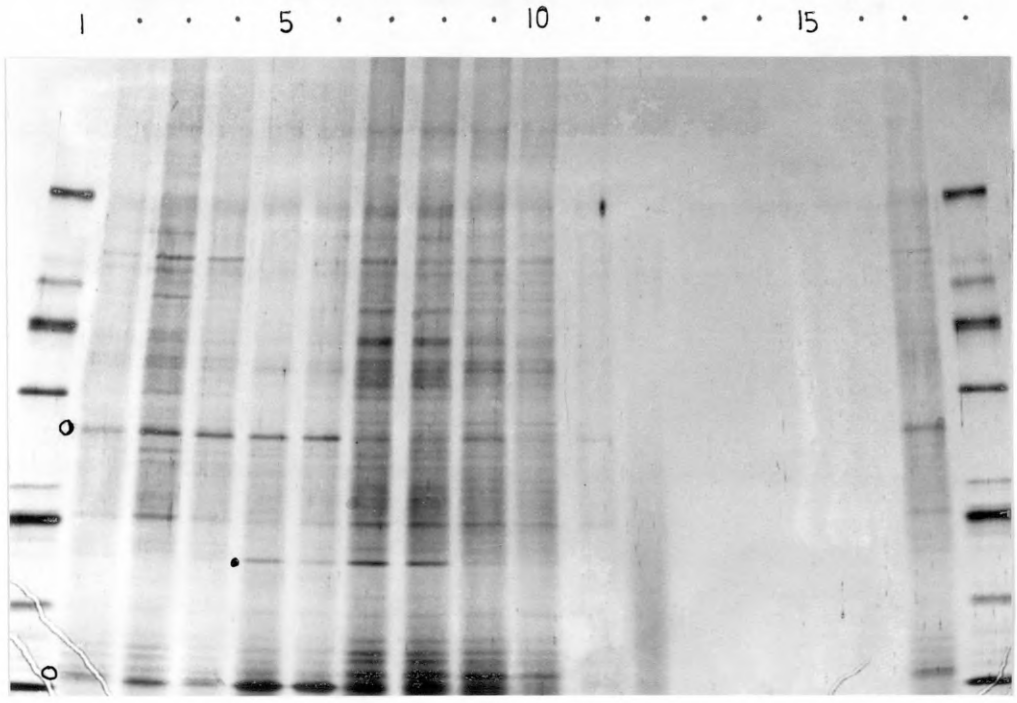


Plate 1 contains 5% malt extract broth (2.36% TN).
Plate 2 contains medium with 2.36% TN.
Plate 3 contains medium with 0.127% TN.
Plate 4 contains medium with 0.03% TN.
Plate 5 contains medium with 0.0% TN.

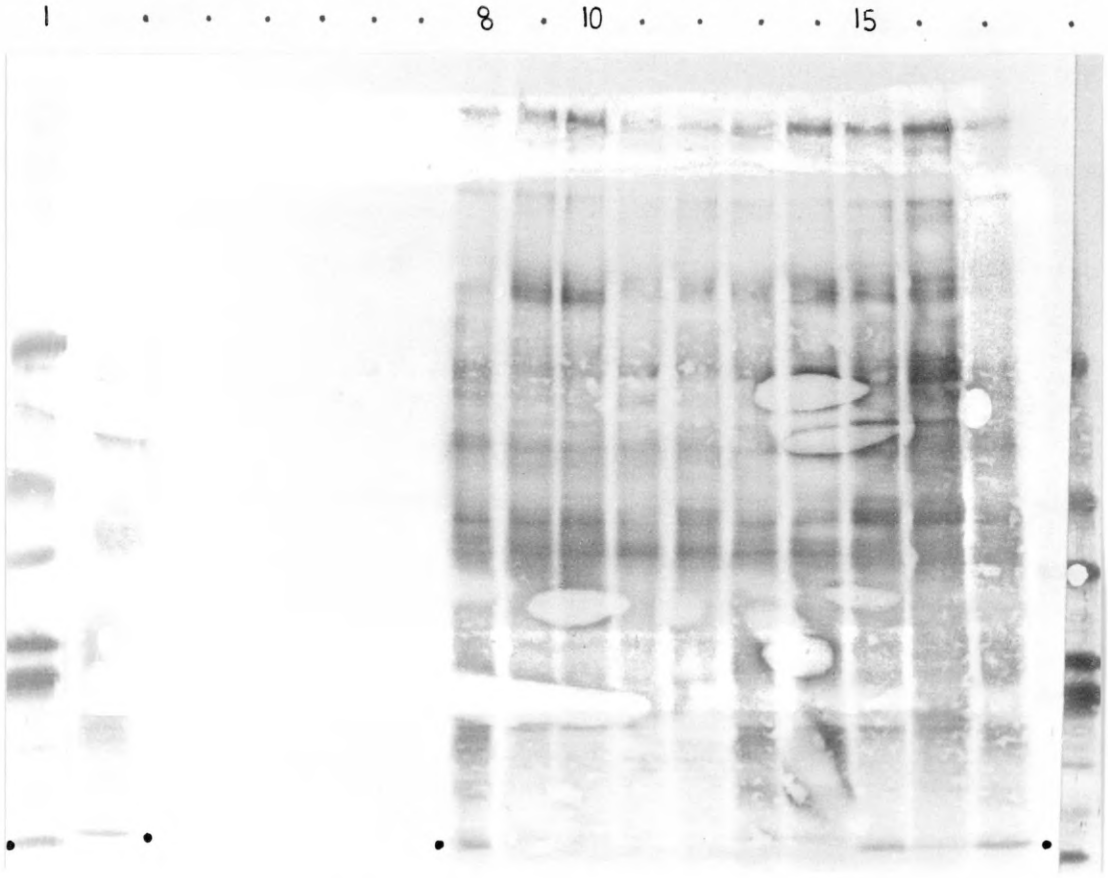
Figure 28: SDS-PAGE/silver stain analysis of S. lacrymans FPRL 12C grown in liquid media containing decreasing amounts of total nitrogen (TN).



o represents proteins which become less major.
. represents new proteins.

Tracks 1 and 18 represent low molecular weight standard proteins.
Tracks 2 and 17 represent standard mycelium of S. lacrymans FPRL 12C.
Tracks 3 - 12 represent S. lacrymans FPRL 12C mycelium cultured in 5% MEB (3, 4); 2.36% TN (5, 6); 0.127% TN (7, 8); 0.03% TN (9, 10) and 0.0% TN (11, 12).
Tracks 13 - 16 represent the culture media after growth of S. lacrymans FPRL 12C shown in tracks 3 - 12. The following tracks represent media containing 2.36% TN (13); 0.127% TN (14); 0.03% TN (15) and 0.0% TN (16).

Figure 29: Western blot analysis of antigens of S. lacrymans FPRL 12C grown in liquid medium containing decreasing amounts of total nitrogen (TN).



Tracks 1 and 18 represent low molecular weight standard proteins.

Tracks 2, 8 and 17 represent standard mycelium of S. lacrymans FPRL 12C.

Tracks 3 - 7 represent medium after culture of S. lacrymans FPRL 12C in 0.0% TN (3); 0.03% TN (4); 0.127% TN (5); 2.36% TN (6) and 5% MEB (7).

Tracks 9 - 16 represent S. lacrymans FPRL 12C cultured in 0.03% TN (9, 10); 0.127% TN (11, 12); 2.36% TN (13, 14) and 5% MEB (15, 16).

. indicates antigen 19.

which is present in standard mycelium only (Figure 29, tracks 15 - 17).

The protein similarity index (Table 13) confirms the absence of detectable proteins in mycelium cultured in 0% TN medium; illustrates that there is a difference between the proteins in mycelium cultured in different media containing similar amounts of TN, viz 5% MEB and 2.36% TN; and indicates that the banding pattern changes as the TN content of the medium decreases. In addition, Table 13 indicates that there is a relatively high degree of similarity between mycelium of S. lacrymans cultured in media containing different amounts of TN; and that the protein similarity index is more like that established for bona fide isolates of S. lacrymans FPRL 12C (Table 8) than that for non-S. lacrymans species (Table 9), e.g. in 0.03% TN a similarity of 74.7 was obtained which is markedly higher than the 43.5 obtained for the non-S. lacrymans isolate which was most similar to S. lacrymans (Table 9).

6.4 The harvest of different regions of mycelium.

Three separate uplifts of young and aged mycelium of S. lacrymans FPRL 12C were analysed by SDS-PAGE/silver staining and western blotting to determine if the protein and antigenic profiles of standard mycelium of S. lacrymans FPRL 12C was representative of all parts of laboratory isolated mycelium. The protein profiles are shown in Figure 30 and indicate, firstly, that there is a difference in profile between standard, young and aged mycelium and, secondly, that profiles are similar

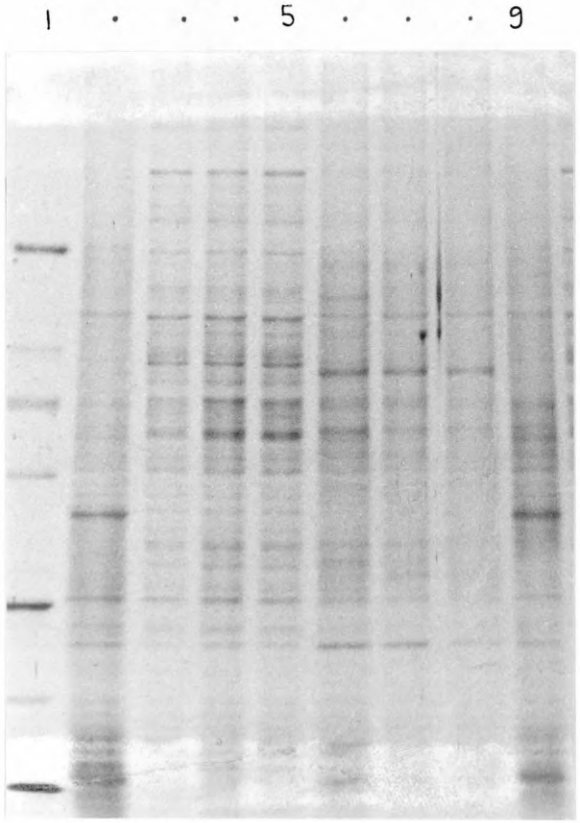
Table 13: Protein similarity index of mycelium of *S. lacrymans* FPRL 12C cultured in media containing differing amounts of total nitrogen (TN).

Components of medium	% TN	Percentage similarity	
Malt extract broth (5%)	2.36	98	(4)
Mycological peptone/dextrose	2.36	85.1	(4)
" " "	0.127	67.9	(3)
" " "	0.03	74.7	(4)
Dextrose	0.0	*	(2)

* indicates too few proteins for analysis.

( ) indicates number of analyses.

Figure 30: SDS-PAGE/silver stain analysis of young and aged mycelium of S. lacrymans FPRL 12C.



Track 1 represents low molecular weight standard proteins.

Tracks 2 and 9 represent standard mycelium of S. lacrymans FPRL 12C.

Tracks 3 - 5 represent 3 separate uplifts of young mycelium of S. lacrymans FPRL 12C.

Tracks 6 - 8 represent 3 separate uplifts of aged mycelium of S. lacrymans FPRL 12C.

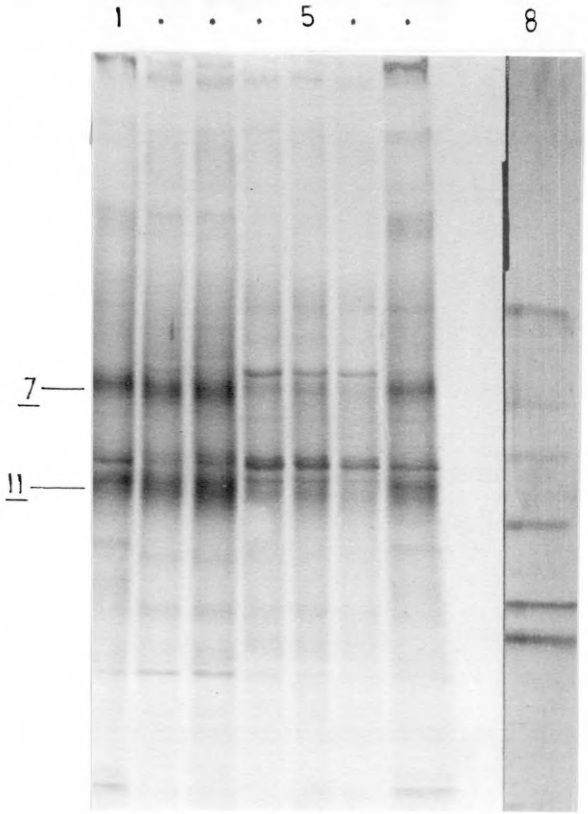
between uplifts of each type of mycelium, e.g. compare tracks 3 - 5. Young and aged mycelium had protein percentage similarities of 72.1 and 64.4 respectively.

The result of a western blot analysis (Figure 31) indicates that the differences between standard, young and aged mycelium make for instant discrimination between each type of mycelium in spite of the similarities which can be observed between standard and either young or aged mycelium. Young mycelium notably possesses relatively well defined major antigens 6, 9 and 10 (tracks 4 - 6) which are absent in aged mycelium (tracks 2 and 3). The major antigens in aged mycelium are antigens 15, 7 and 11; the last two, in common with most antigens of aged mycelium, being represented by diffuse bands. Antigens from both young and aged mycelium could be found in the standard preparation of FPRL 12C, e.g. antigen 10, and possibly 2, 7, 9 and 11, were common to young and standard mycelium in Figure 31 but other experiments revealed the presence also of antigens 6 and 9 in standard mycelium; whilst antigens 1, 7, 11, and possibly 2, 3, 4, 5, 15, were common to standard and aged mycelium.

6.5 The effect of culture at 40°C.

SDS-PAGE/silver staining and western blotting were used to analyse *S. lacrymans* and *S. himantioides* which had been cultured for different periods of time at 40°C in order to investigate the effect of temperature on *S. lacrymans*. Visual observations of SDS-PAGE/silver stained gels (results not shown) indicated that changes

Figure 31: Western blot analysis of young and aged mycelium of S. lacrymans FPRL 12C.



Track 8 represents low molecular weight standard proteins.

Tracks 1 and 7 represent standard mycelium of S. lacrymans FPRL 12C.

Tracks 2 and 3 represent 2 separate uplifts of aged mycelium.

Tracks 4 - 6 represent 3 separate uplifts of young mycelium.

. represents antigens 6, 9 and 10.

7, 11 represent antigens 7 and 11.

had occurred which are analysed in Table 14. This illustrates that after 6 hours at 40°C the only proteins which had been lost were in aged mycelium of S. himantioides. The major changes observed in both organisms after 24 hours at 40°C indicate that proteins of young and aged mycelium of S. himantioides are probably more resistant to exposure to 40°C than those of S. lacrymans; and, whilst young mycelium of S. himantioides is probably more resistant to 40°C than aged mycelium, the two different morphological regions of S. lacrymans are equally affected. The results in Table 14 also indicate that the viability of S. lacrymans is affected after >1 hour at 40°C, whereas S. himantioides is not affected till >6 hours at 40°C. One new protein was recorded at 38 kDa in young mycelium of S. lacrymans after 24 hours incubation at 40°C.

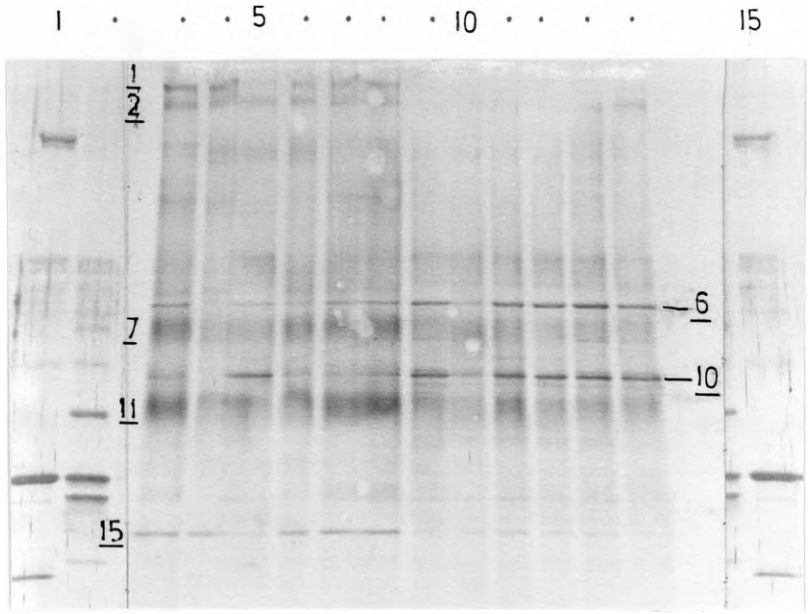
Western blot results of S. lacrymans (Figure 32) and S. himantioides indicated that it was possible to detect antigens in both young and aged mycelium after all incubation periods. In general, the results produced by western blotting confirmed conclusions reached after SDS-PAGE/silver staining, viz major changes in profile for both young and aged mycelium of both species were only noticeable after 24 hours incubation at 40°C, e.g. Figure 32, tracks 4 and 10. Antigens 6 and 10 are especially prominent in young mycelium of S. lacrymans (tracks 11 - 14) but after 24 hours at 40°C (track 10) there is a loss of prominence of these antigens, with the result that the profile bears a closer resemblance to aged mycelium than young mycelium, e.g. compare tracks 6 and 10. Aged mycelium of S. lacrymans shows little change over the 0 - 6 hour incubation periods

Table 14: The effect of incubation at 40°C on the viability and the proteins detected by SDS-PAGE/silver staining of agar grown mycelium of S. lacrymans FPRL 12C and S. himantioides.

Period at 40°C (hours)	Viability		Percentage loss in proteins detected by SDS-PAGE/s. stain			
	S. lacrymans	S. himantioides	S. lacrymans		S. himantioides	
			Young	Aged	Young	Aged
0	+	+	0	0	0	0
1	+	+	0	*	0	0
4	-	+	0	*	0	0
6	-	+	0	0	0	71.4
24	-	-	87.2	93.6	47.6	76.9
24	New proteins (kDa)		38	-	-	-

* indicates insufficient mycelium for analysis.
\+ " viable after subculture.
\- " not viable after subculture.

Figure 32: The effect of incubation periods at 40°C on the antigens of agar grown S. lacrymans FPRL 12C.



Tracks 1, 2 and 15 represent molecular weight standard proteins.
Tracks 3 - 8 represent aged mycelium.
Tracks 9 - 14 represent young mycelium.

Incubation periods are as follows:
0 hours - tracks 3, 8, 9 and 14.
1 hour - tracks 7 and 13.
4 hours - tracks 6 and 12.
6 hours - tracks 5 and 11.
24 hours - tracks 4 and 10.

Antigens are represented thus 1, 2 etc.

(tracks 5 - 8) but after 24 hours at 40°C (track 4) antigens 6 and 10 are absent; and the major antigenic representatives are antigens 1, 2, 7, 11 and 15.

6.6 The effect of Trichoderma.

S. lacrymans FPRL 12C which had been exposed to three species of Trichoderma was analysed by SDS-PAGE/silver staining and western blotting in order to investigate the effect of other fungi upon the molecular profile of S. lacrymans. These organisms had previously been investigated for their effects upon the morphology and viability of S. lacrymans (A. Score, personal communication). The protein similarity indices are shown in Table 15 which indicates that only T. harzianum affected the protein profile of S. lacrymans. Figure 33 additionally illustrates that the effect was manifest only in young mycelium and only after contact of the two organisms. Figure 33 (tracks 7 and 8) also shows the appearance of a new protein, 23 kDa, 24 hours after contact of T. harzianum and S. lacrymans. Western blot results indicated that the antigens of aged mycelium of S. lacrymans were not affected by exposure to either T. longibrachiatum (Figure 34, tracks 3 - 8) or T. saturnisporum over the incubation periods tested. However, T. harzianum (Figure 35) caused loss of antigen 15, and possibly 13 and 14, 4 days after contact of the organisms (track 3). Young mycelial antigens were affected after contact with all three Trichoderma species. T. longibrachiatum and T. saturnisporum affected S. lacrymans in the same way, viz progressive loss of antigens 6, 9 and 10 (Figure 34, tracks 11 - 13)

Table 15: Protein similarity index of agar grown mycelium of S. lacrymans FPRL 12C after exposure to 3 species of Trichoderma.

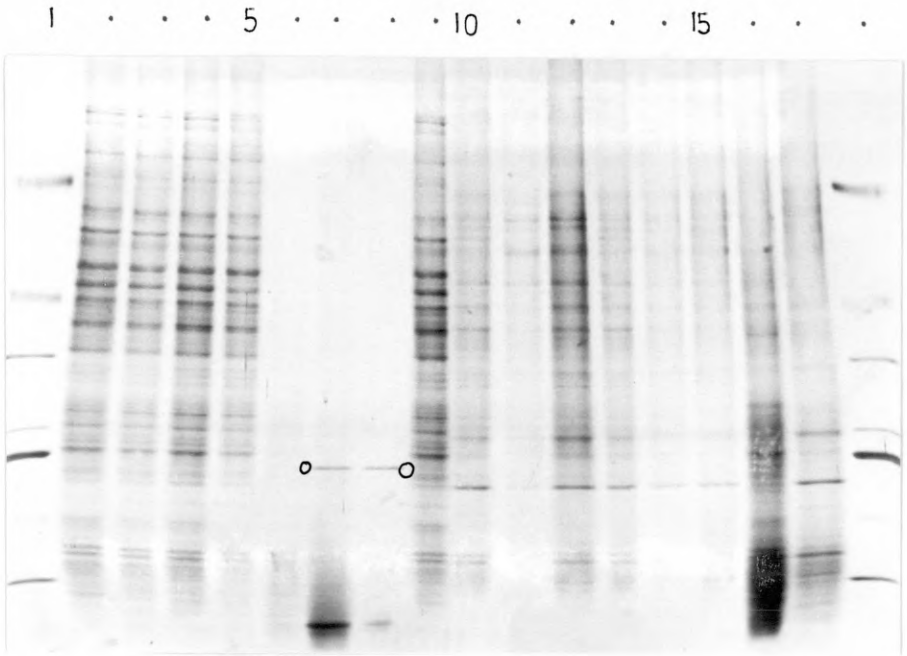
Period of exposure (days)	Exposure organisms							
	S. lacrymans		T. harzianum		T. s'sporum		T. long'um	
	Young	Aged	Young	Aged	Young	Aged	Young	Aged
0	100	100	100	100	100	100	100	100
1	100	100	100	99	100	100	100	100
2	100	100	100	98	100	100	100	100
3	100	100	*	99	99	100	99	99
4	100	100	*	97	100	98	97	99
7	100	100	*	100	98	98	100	98

* indicates too few proteins for analysis.

T. s'sporum indicates T. saturnisporum

T. long'um indicates T. longibrachiatum

Figure 33: The effect of T. harzianum on the SDS-PAGE/silver stained profile of S. lacrymans FPRL 12C.

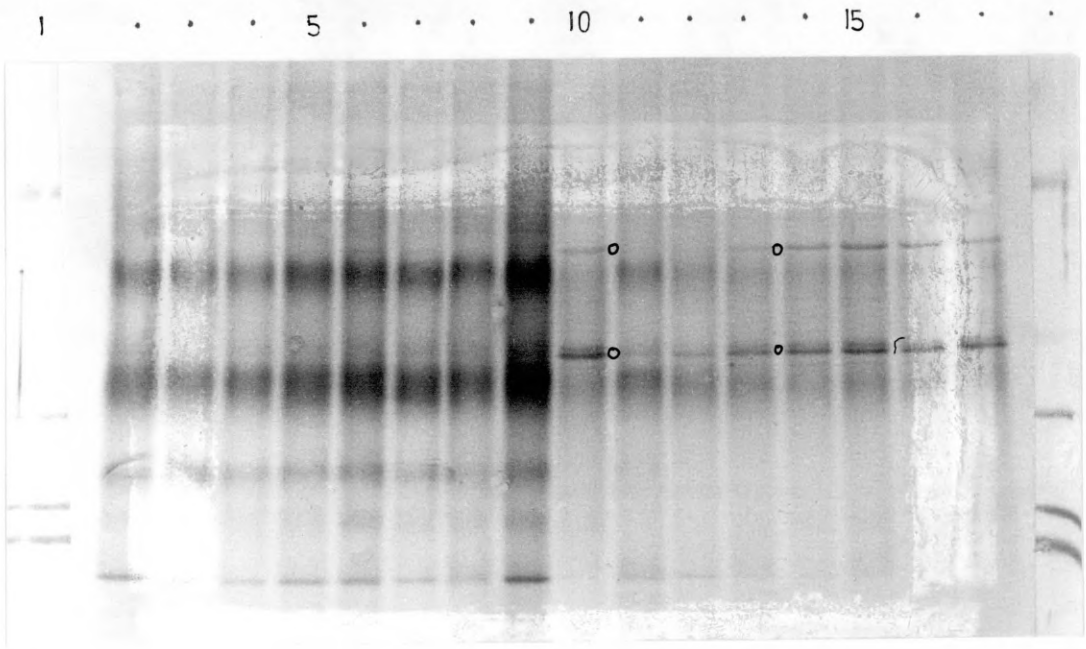


Tracks 1 and 18 represent low molecular weight standard proteins.
Tracks 2 - 9 represent young mycelium of agar grown S. lacrymans FPRL 12C.
Tracks 10 - 17 represent aged mycelium of agar grown S. lacrymans FPRL 12C.

Incubation periods after exposure to T. harzianum are as follows:
0 days - tracks 3 and 11;
1 day - tracks 4 and 12;
2 days - tracks 5 and 13;
3 days - tracks 6 and 14;
4 days - tracks 7 and 15;
7 days - tracks 8 and 16.

o indicates novel protein.

Figure 34: The effect of T. longibrachiatum on the antigenic profile of S. lacrymans FPRL 12C.

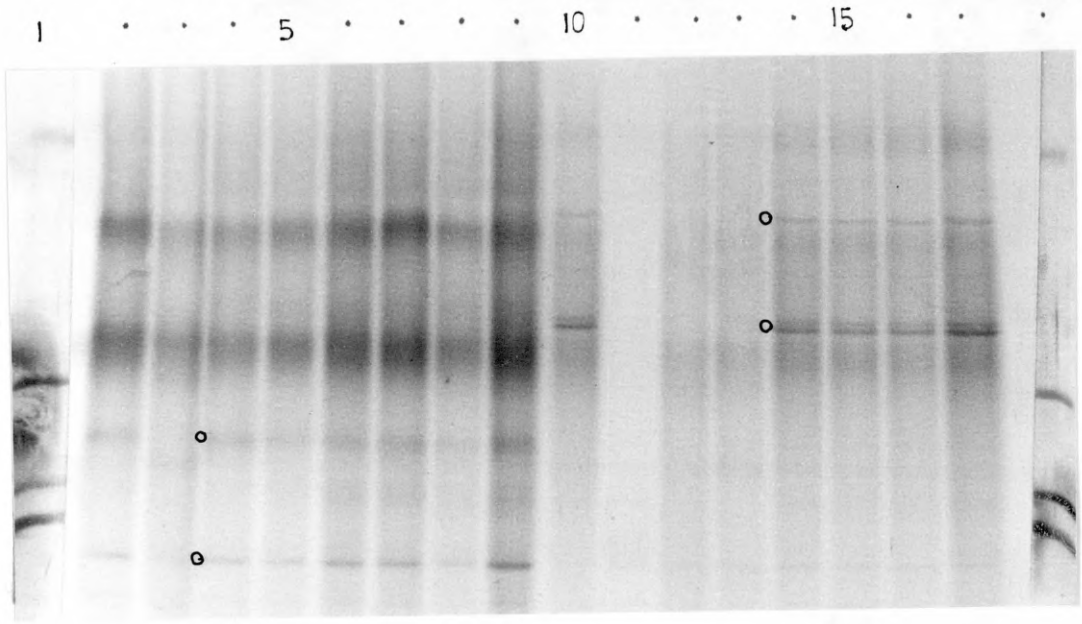


Tracks 1 and 18 represent low molecular weight standard proteins.
Tracks 2 - 9 represent aged mycelium of S. lacrymans FPRL 12C.
Tracks 10 - 17 represent young mycelium of S. lacrymans FPRL 12C.

Incubation periods with T. longibrachiatum are as follows:
0 days - tracks 2, 9, 10 and 17;
1 day - tracks 7 and 15;
2 days - tracks 6 and 14;
3 days - tracks 5 and 13;
4 days - tracks 4 and 12;
7 days - tracks 3 and 11.

o indicates antigens lost after fungal contact.

Figure 35: The effect of T. harzianum on the antigenic profile of S. lacrymans FPRL 12C.



Tracks 1 and 18 represent low molecular weight standard proteins.
Tracks 2 - 9 represent aged mycelium of S. lacrymans FPRL 12C.
Tracks 10 - 17 represent young mycelium of S. lacrymans FPRL 12C.

Incubation periods with T. harzianum are as follows:

0 days - tracks 2, 8, 10 and 16;
1 day - tracks 7 and 15;
2 days - tracks 6 and 14;
3 days - tracks 5 and 13;
4 days - tracks 4 and 12;
7 days - tracks 3 and 11.

o indicates antigens lost after fungal contact.

until the antigenic profile was more similar to aged mycelium than young mycelium, e.g. compare track 11 with track 3. However, T. harzianum caused loss of antigens from the antigenic profile of S. lacrymans FPRL 12C (Figure 35, tracks 12 and 13) resulting in absence of reactivity 4 days after contact of the organisms (track 11).

6.7 Discussion.

6.7.1 Basic parameters of growth.

In the studies reported in chapters 3 - 5 S. lacrymans was grown up in liquid culture because standardisation of culture methods was consistent with molecular methods and gave reproducible results. Work in this chapter indicated that similar and consistent electrophoretic and antigenic patterns were obtained for standard and agar grown mycelium of S. lacrymans FPRL 12C during numerous subcultures over the course of 6 months. The minor differences found between protein banding patterns of standard mycelium and agar grown material may be associated with enzymes loosely attached to the exterior of the unwashed mycelium and concerned with substrate utilisation. The similarity in antigenic profile between standard and agar grown mycelium probably reflects the nature of the immunogen which was prepared from standard, i.e. washed, mycelium. Molecules associated with external functions of the mycelium would not be identified by the present antiserum which would detect only immunogenic molecules associated with either the walls or contents of hyphae. The present antiserum indicates that variation of basic cultural parameters

does not cause gross changes associated with the basic structural and 'housekeeping' molecules but it is no pointer to changes directly associated with exploitation of the environment.

6.7.2 Variation in TN.

The absence of antigens in media containing differing amounts of TN probably reflected the nature of the immunogen. The lack of proteins in the media might indicate either absence of proteins or, more probably, lack of detection due to an insufficiently sensitive technique since it is known that filamentous fungi secrete large amounts of protein into the medium, e.g. Wosten et al. (1991) and Sprey (1988).

The direct relationship between the lushness of growth of S. lacrymans and the numbers of proteins detected in mycelium is probably also related to the TN content of the medium since vigour of growth of all organisms is related to nutrients present in the growth medium. Carter & Lynch (1991) found that the SDS-PAGE/silver stained profile of T. harzianum varied according to whether the carbon source in the medium was provided by glucose or straw; and M^{ac}Donald, Liwicki & Broda (1984) found changes in the electrophoretic pattern of intracellular proteins of Phanerochaete chrysosporium, a white rot Basidiomycete, with the appearance of 20 novel proteins and the disappearance of other proteins when in conditions of nitrogen starvation. MEB contains vitamins, amino acids and other growth factors (Oxoid, personal communication) which are probably responsible for the differences in morphology and protein percentage similarity of mycelium cultured in the two different

media containing 2.36% TN. Normal lab medium has a C:N ratio of 32:1 (Levi & Cowling, 1969) and differences observed between the protein profiles of S. lacrymans in the other media could be related solely to falling nitrogen levels and concomitant alteration in the C:N ratios.

Wood contains 0.03 - 0.127% nitrogen (Deacon, 1984) and, since some of the proteins observed in mycelium cultured in media containing >0.127% TN may be associated with excess amounts of nitrogen, the morphology and molecular nature of mycelium cultured in media containing either 0.03% TN or 0.127% TN may be more representative of the organism in its natural environment. Standard mycelium, therefore, may not be the most appropriate reference material for investigation of SDS-PAGE/silver stained profiles of the organism in the wild and it may be that antigenic profiles have greater potential for this purpose since they were found to be similar regardless of TN availability. Antigen 19, present only in mycelium from media containing MEB, may be associated with nutrients other than the nitrogen content of the medium. Lack of detectable proteins in the sparse growth from inocula in media containing 0% TN could be attributable to growth furnished by autolysis of limited cytoplasmic sources of nitrogen.

SDS-PAGE/silver staining also indicated that all mycelium of S. lacrymans shared a basic protein banding pattern provided that nitrogen was present in the medium. This is perhaps indicative of the ability of S. lacrymans to extract and conserve nitrogen when growing in media with low nitrogen levels (Watkinson, Davison &

Bramah, 1981). It was shown that the amount of nitrogen in mycelium of S. lacrymans was 43 - 53 times higher than in the wood from which it had grown. Most wood species have a C:N ratio of 350 - 500:1 (Cowling & Merrill, 1966) and consequently there is a critical shortage of nitrogen in wood. Extraction and conservation of nitrogen is an adaptation to growth in wood which is not confined to S. lacrymans, e.g. Staszczak & Nowak (1984) found little change in the proteinase pattern of C. versicolor in response to nitrogen starvation. It is known that nitrogen is recycled by S. lacrymans in older regions of mycelium (Watkinson, 1975) and this organism, like C. versicolor in conditions of low nitrogen (Levi & Cowling, 1969), may preferentially allocate nitrogen to essential metabolic processes, e.g. the production of nucleic acids and extracellular enzymes.

6.7.3 Young and aged mycelium.

Antigenic profiles revealed differences between young and aged mycelium which indicated that these may represent two different morphological types of mycelium. This is supported by the observations of Hornung & Jennings (1981) who listed 4 stages in the development of surface mycelium from an inoculum of S. lacrymans. Young mycelium may correspond to stage II which is characterised by 'fur-like white mycelia appearing on the substrate surface', whilst aged mycelium probably corresponds to the 'macroscopically visible syrrotia' which are characteristic of stage III.

Morphological types vary in structure and function. Young mycelium represents the region of the hypha which

is chiefly concerned with extension and exploitation of the environment, consequently it has thinner walls with fewer layers than aged mycelium (Hunsley & Burnett, 1970) and a cytoplasm extensively concerned with peripheral enzyme production (Pugh & Cawson, 1977). Aged mycelium is more highly vacuolated, with cytoplasmic activities concerned with recycling of nutrients by autolysis (Watkinson, 1975). It is probable, therefore, that the antigenic differences of young and aged mycelium are related to differences in structural or cytological functions of these two regions of mycelium. In support of this, Marchant & Smith (1968) used antisera raised against tips or mature hyphae of Fusarium culmorum to demonstrate immunofluorescent differences between tips and mature hyphae which were attributed to wall structure or cytoplasmic activities. Different antigenic species and profiles may be associated with the other growth phases of S. lacrymans and these could provide information on the molecular processes associated with hyphal differentiation.

The similarity demonstrated between liquid and agar grown profiles allowed the direct comparison of young and aged mycelium with standard mycelium and indicated that young and aged mycelium had more antigens in common with standard FPRL 12C than with each other. This comparison illustrated that the representative antigenic profile of S. lacrymans FPRL 12C standard mycelium is composed of antigens associated with either young or aged mycelium. For example, two diffuse antigens, 7 and 11, at 51 - 54.5 and 41.5 - 42.5 kDa respectively, are the major antigens in aged mycelium of S. lacrymans FPRL 12C; and the well defined antigens 6, 9 and 10 (at 57,

44 and 43 kDa respectively) are associated with young mycelium. This antigenic makeup of standard mycelium is a logical outcome in view of its morphological composition. The variation observed in the frequency with which antigens were found to occur in the antigenic profile of standard mycelium of S. lacrymans FPRL 12C is related to the morphological composition of the standard mycelium selected for extraction.

6.7.4 Exposure to 40°C.

The temperature optima of S. lacrymans and S. himantioides, 22°C and 34°C respectively, indicated that S. lacrymans would be more sensitive to the effect of heat than S. himantioides. This was confirmed by viability studies, alteration of profile and loss of moleclar species in young and aged mycelium. The greater loss of proteins in aged mycelium indicated that the proteins in young mycelium are perhaps more resistant to heat. Since the young mycelium initially responds to the environment it might be that proteins in young mycelium are necessarily more robust than those in aged mycelium. Alternatively, since the process of autolysis is ongoing in aged mycelium it is possible that additional degradative changes caused by exposure to lethal temperatures would be noticed in this region first. Since young mycelium showed a greater resemblance to aged mycelium after 24 hours at 40°C, and aged mycelium progressively lost antigens over the same time period, it is possible that antigenic profiles indicated successive stages in senescence. The new protein recorded in young mycelium of S. lacrymans after 24 hours exposure to 40°C may be a useful indicator of senescence. It could be that either absence of the well

defined antigens associated with young mycelium or the presence of a novel protein at 38 kDa in a SDS-PAGE/silver stained profile would confirm non-viability of S. lacrymans after use of the control method which involves heating an infected building (Koch, 1991).

6.7.5 Exposure to Trichoderma.

Viability and morphological studies on interactions between S. lacrymans and 3 species of Trichoderma had established that exposure to T. harzianum killed S. lacrymans, whereas T. longibrachiatum was killed by overgrowth of S. lacrymans and T. saturnisporum caused cessation of growth in both organisms (A. Score, personal communication). The protein and antigenic profiles of S. lacrymans reflected these biological interactions: T. longibrachiatum and T. saturnisporum caused the loss of defined antigens from the antigenic profile of young mycelium with subsequent resemblance to the aged profile, and only T. harzianum caused loss of all proteins and antigens after contact of the organisms. T. harzianum exerted its preliminary effect upon the point of contact of the two organisms, viz young mycelium. The novel protein at 23 kDa and the major antigens associated with aged mycelium may be indicative of senescence and could be exploited in an assessment of viability which would indicate the success of potential biological control measures. In this context it would be of interest to monitor whether overgrowth of T. longibrachiatum by S. lacrymans were ultimately accompanied by recovery of the well defined antigens associated with young mycelium.

6.7.6 Summary.

Work in previous chapters strived to investigate material cultured under standardised conditions. This was useful for the delineation of molecular profiles for isolate identification but gave no indication of the potential range of molecular response to environmental factors which would apply in uncontrolled field conditions: the situation where organism identification is of greatest importance.

Protein similarity indices for results in this chapter indicated that alteration in laboratory cultural conditions produced a large degree of variation in the established molecular profile of mycelium, e.g. mycelium harvested as young (72.1), aged (64.4) or from different culture conditions (98 - 100, basic cultural parameters; 68.4 - 100, TN variation). However, this variation occurred within defined limits which corresponded with the protein similarity index established for S. lacrymans isolates (68.4 - 100) rather than that established for non-S. lacrymans species (0.0 - 43.5). With the exception of young and aged mycelium, antigenic profiles showed little variation from that of standard mycelium. However, the profiles of young and aged mycelium could be related to that of standard mycelium. Therefore the protein and antigenic similarity indices emphasised that there are still sufficient similarities between mycelium cultured under less controlled laboratory conditions and standard mycelium of S. lacrymans FPRL 12C to facilitate identification of mycelium cultured under less controlled laboratory conditions.

Protein and antigenic similarities are probably related to a set of crucial proteins and antigens which are unaffected by environment, e.g. housekeeping and structural proteins. Further investigation of the changes which either were initiated by potentially lethal culture conditions, e.g. the new proteins which were observed after exposure to 40°C and T. harzianum, or were demonstrated to be associated with the morphological stage of the organism, e.g. young and aged mycelium, could lead to identification of markers for the metabolic state of S. lacrymans. These metabolic markers could be exploited for assessment of viability of field samples, especially young mycelium, both before and after remedial treatment.

CHAPTER 7. FURTHER ANALYSES OF LABORATORY CULTURES OF *S. lacrymans*.

7.1 General introduction.

Work in previous chapters has indicated some areas where further investigation was required. Firstly, marked differences in the antigenic nature of two morphological forms of mycelium of S. lacrymans, viz young and aged mycelium, were indicated (chapter 6). However, the relationship between the major antigens of these morphological forms was not clear and it is possible that the major antigens of aged mycelium were derived from those of young mycelium either by sample preparation or by protein processing during differentiation. Secondly, the anomaly of the relatively low protein percentage similarity of isolate BF-050 to standard mycelium of S. lacrymans FPRL 12C had previously raised doubts about the identification of this organism as S. lacrymans (chapter 5) and an alternative morphological form of mycelium, young mycelium, could possibly resolve the identity of this isolate. Thirdly, decay of wood by S. lacrymans involves an early stage of colonisation, a period of active decay and a stage when autolysis of mycelium occurs after useful nutrients have been exhausted. These stages may correspond with distinct morphological forms of S. lacrymans, e.g. young and aged mycelium (chapter 6), which possibly could be detected by present analytical techniques.

The main objectives of work in this chapter were

1. To investigate whether the major antigens of aged mycelium of S. lacrymans FPRL 12C can be derived by sample preparation from the major antigens of young mycelium.

2. To use Con A to further investigate the major antigens of young and aged mycelium.
3. To investigate the use of an alternative reference mycelium, young mycelium, in the identification of the anomalous isolate of S. lacrymans, BF-050.
4. To investigate the SDS-PAGE/silver stain and western blot profiles of S. lacrymans FRPL 12C grown in pine and lime sapwood blocks.

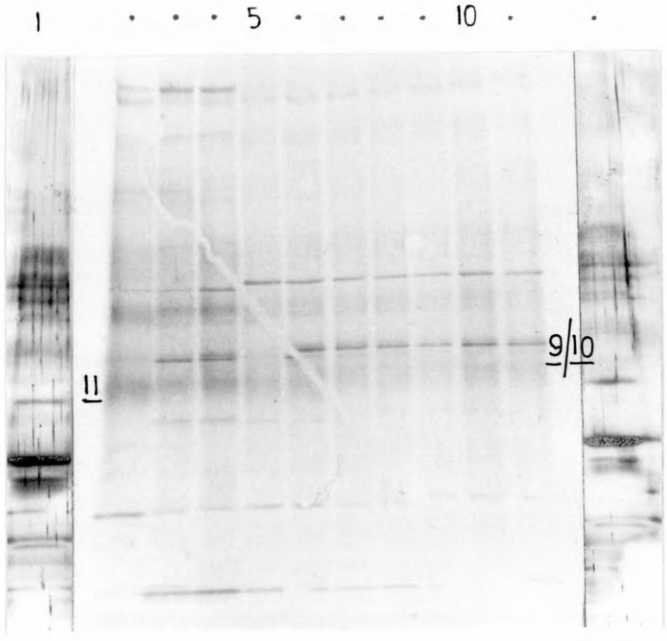
7.2 Sample preparation and the major antigens of young mycelium.

Samples of young mycelium were ground to a slurry in PBS and incubated at R/T for varying periods prior to addition of boiling mix at either R/T or 4°C in order to investigate whether the major antigens of aged mycelium of S. lacrymans FPRL 12C were an artefact related to sample preparation. Figure 36 shows the results of analysis of this material by western blotting and indicates that only incubation for 24 hours at R/T in PBS (track 5) had any effect on the antigenic profile. Only antigens 9 and 10 (43 and 44 kDa respectively) were affected by incubation at R/T, with no concomitant darkening of antigen 11 (compare tracks 5 and 6) to the density of staining illustrated in aged mycelium (track 2) or standard mycelium (tracks 4).

7.3 Con A staining of young and aged mycelium.

Further investigation of the nature of the major antigens in the profiles of young and aged mycelium of

Figure 36: The effect of sample preparation on the major antigens of young and aged mycelium of S. lacrymans FPRL 12C.



Tracks 1 and 12 represent low molecular weight standard proteins.
Track 2 represents aged mycelium of S. lacrymans FPRL 12C.
Tracks 3 and 4 represent standard mycelium of S. lacrymans FPRL 12C.
Tracks 5 - 10 represent young mycelium of S. lacrymans incubated at R/T in PBS for the following periods prior to addition of boiling mix: 24 hours (track 5); 1 hour (track 6); 30 min (track 7); 15 min (track 8); 5 min (track 9) and 0 min (track 10).
Track 11 represents a sample prepared at 4°C in PBS:boiling mix (2:1).

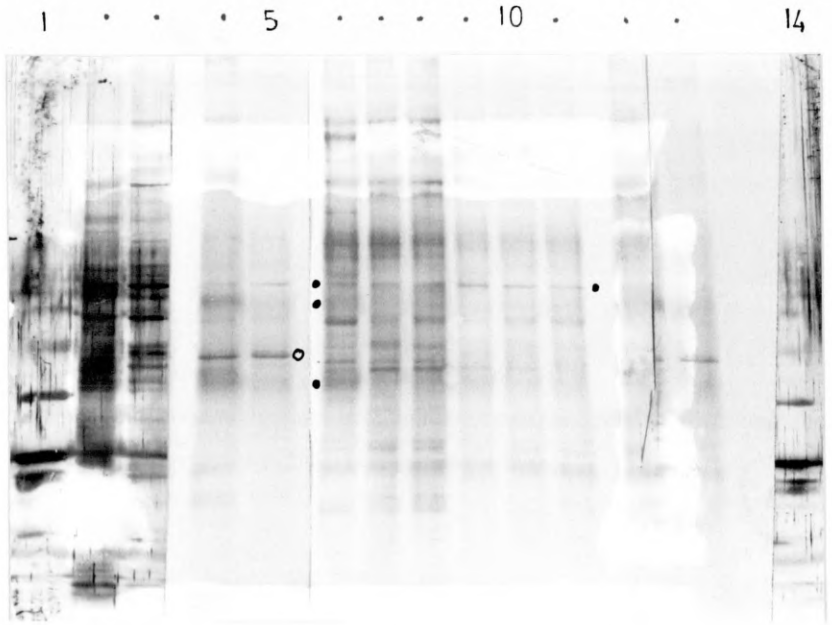
Antigens are indicated thus 9 etc.

S. lacrymans FPRL 12C involved comparison of immunostained profiles of standard mycelium with electroblotted profiles of young, aged and standard mycelium subsequently stained with Con A (Figure 37). The glycoprotein nature of one of the major antigens of young mycelium, antigen 6 (57 kDa) was confirmed, e.g. compare young mycelium in track 3 (India ink stain) and track 5 (immunostained) with tracks 9 - 11 (Con A stained). The major antigens associated with aged mycelium, diffuse antigens 7 and 11, are confirmed as glycoproteins, e.g. compare tracks 4 (standard mycelium, immunoblotted) with track 6 (aged mycelium, Con A stained). These results additionally indicate that the two other major antigens of young mycelium, 43 kDa and 44 kDa, are not stained by Con A, e.g. compare track 5 with tracks 9 - 11. This experiment confirms the glycoprotein nature of minor antigens 1, 4, 4.25, 5; and, since it additionally indicates that antigens 3, 13, 14 and 15 are glycosylated (tracks 7 and 8), it demonstrates that most antigens in young and aged mycelium are stained by Con A and are likely to be glycoproteins.

7.4 The identity of isolate BF-050.

Young mycelium was used for preparation of test and reference extracts to further investigate the identity of isolate BF-050. The result of SDS-PAGE/silver stain analysis of young mycelium of S. lacrymans isolates is shown in Figure 38 which indicates that, with the exception of isolate BF-015B (track 8), young mycelium of all isolates has a similar protein profile. The

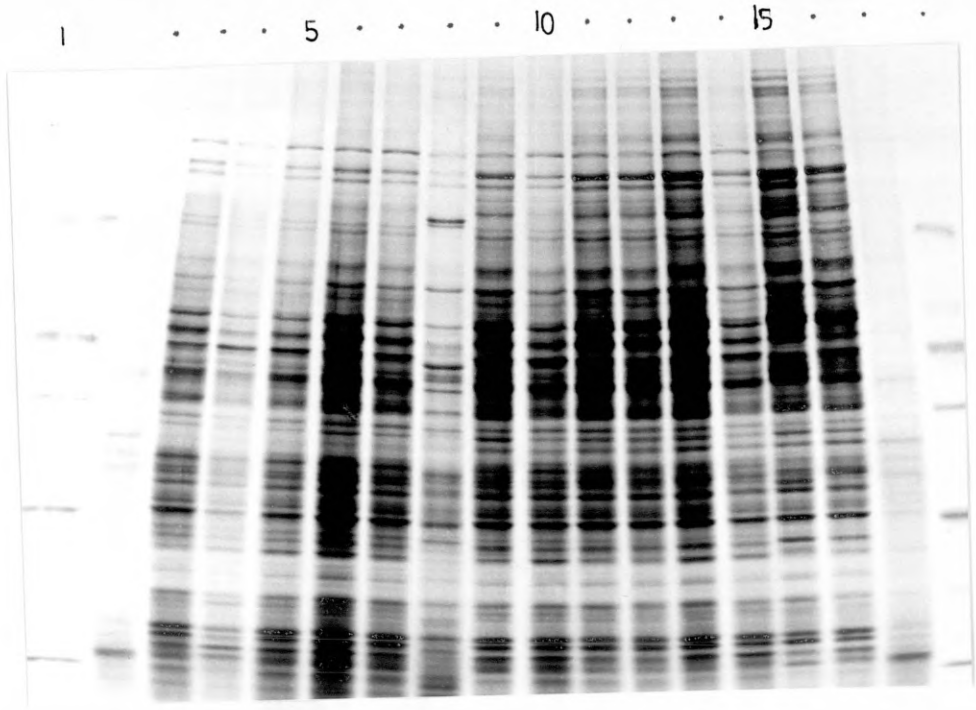
Figure 37: The nature of the major antigens of young and aged mycelium of S. lacrymans FPRL 12C.



Tracks 1 and 14 represent low molecular weight standard proteins.
Tracks 2 and 3 represent India ink stained profiles of standard (track 2) and young (track 3) mycelium of S. lacrymans FPRL 12C.
Tracks 4, 5 and 13 represent immunostaining of standard (track 14) and young (tracks 5 and 13) mycelium of S. lacrymans FPRL 12C.
Tracks 6 - 11 represent Con A staining of aged (track 6), standard (tracks 7 and 8) and young (tracks 9 - 11) mycelium of S. lacrymans.

. represents glycosylated major proteins of young or aged mycelium.
o represents major antigens of young mycelium not stained by Con A.

Figure 38: SDS-PAGE/silver stain analysis of young mycelium of S. lacrymans isolates.



Tracks 1 and 18 represent low molecular weight standard proteins.
Tracks 2 and 17 represent standard mycelium of S. lacrymans FPRL 12C.
Tracks 3 - 16 represent young mycelium of FPRL 12C (3); CMI 152233 (4); BF-01 (5); BF-03A (6); BF-07B (7); BF-015B (8); BF-017B (9); BF-018A (10); BF-023 (11); BF-025 (12); BF-044 (13); BF-046 (14); BF-050 (15) and BF-072 (16).

protein similarity index for young mycelium of S. lacrymans isolates and a few non-S. lacrymans species is shown in Table 16. Western blot analyses of young mycelium are shown in Figure 39 (S. lacrymans isolates) and Figure 40, tracks 9 - 13 (non-S. lacrymans isolates). These indicated that isolate BF-050 had a similar antigenic profile to other S. lacrymans isolates whose profile differed from young mycelium of non-S. lacrymans species. Figure 40 also illustrates that, firstly, there is a difference in definition between antigens of young and aged mycelium of all the wood decay Basidiomycetes analysed, e.g. compare tracks 8 - 13 (young) with tracks 3 - 6 (aged); secondly, the antigenic profiles of standard mycelium of other Basidiomycetes are composed of antigens found in young and aged mycelium, e.g. compare the standard mycelial profile of P. incrassata (Figure 21, track 11) with those of its young and aged mycelium (Figure 40, tracks 10 and 3 respectively).

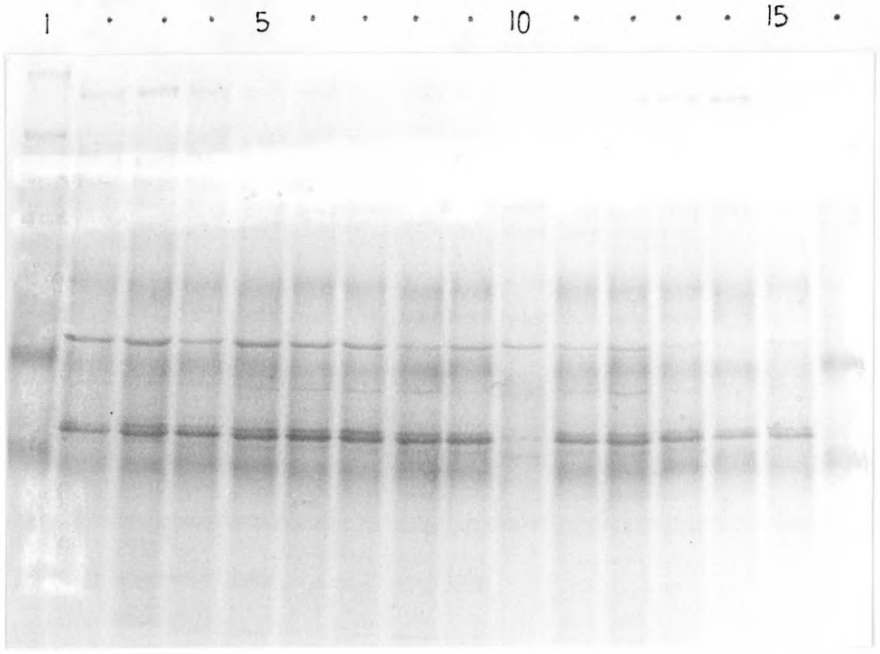
7.5 Analysis of infected wood.

Pine and lime sapwood cubes were decayed to different extents by S. lacrymans FPRL 12C, extracted and subjected to SDS-PAGE/silver stain and western blot analysis to investigate molecular profiles (Figure 41, infected pine sapwood; Figure 42, protein bands associated with lime infected with S. lacrymans FPRL 12C). Both figures indicate the paucity of proteins in uninfected wood (track 3 in both figures) and illustrate a difference of protein profile between standard mycelium and the organism in wood, e.g. compare Figure

Table 16: Protein similarity index of young mycelium of S. lacrymans isolates and some non-S. lacrymans species with reference to young mycelium of S. lacrymans FPRL 12C.

Species	Isolate number	Percentage similarity
S. lacrymans	CMI 152233	100
"	BF-01	100
"	BF-03A	100
"	BF-07B	100
"	BF-017B	90
"	BF-018A	90
"	BF-023	90
"	BF-025	90
"	BF-044	90
"	BF-046	90
"	BF-050	90
"	BF-072	90
"	BF-015B	48.7
P. incrassata		44.0
C. versicolor		41.9
C. puteana		34.3
F. vaillantii		34.0
P. panuoides		29.3

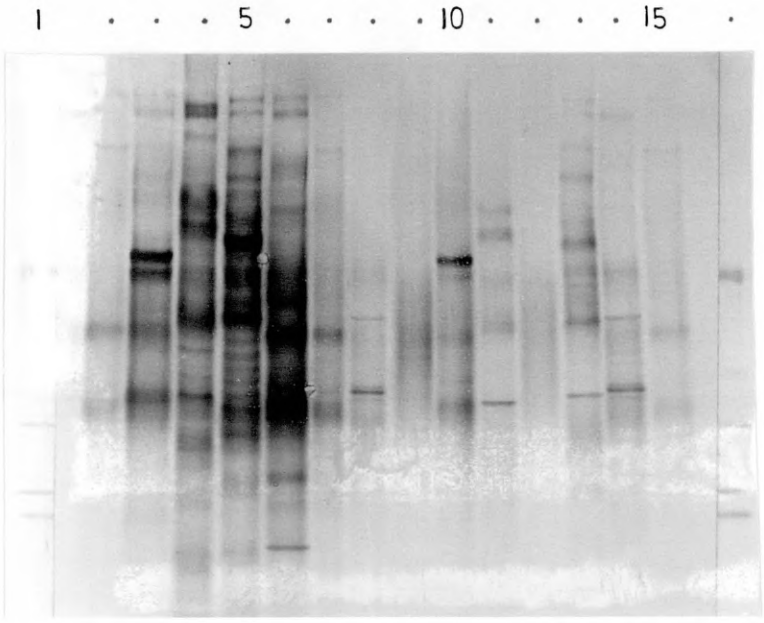
Figure 39: Western blot analysis of young mycelium of S. lacrymans isolates.



Tracks 1 and 16 represent S. lacrymans FPRL 12C standard mycelium.

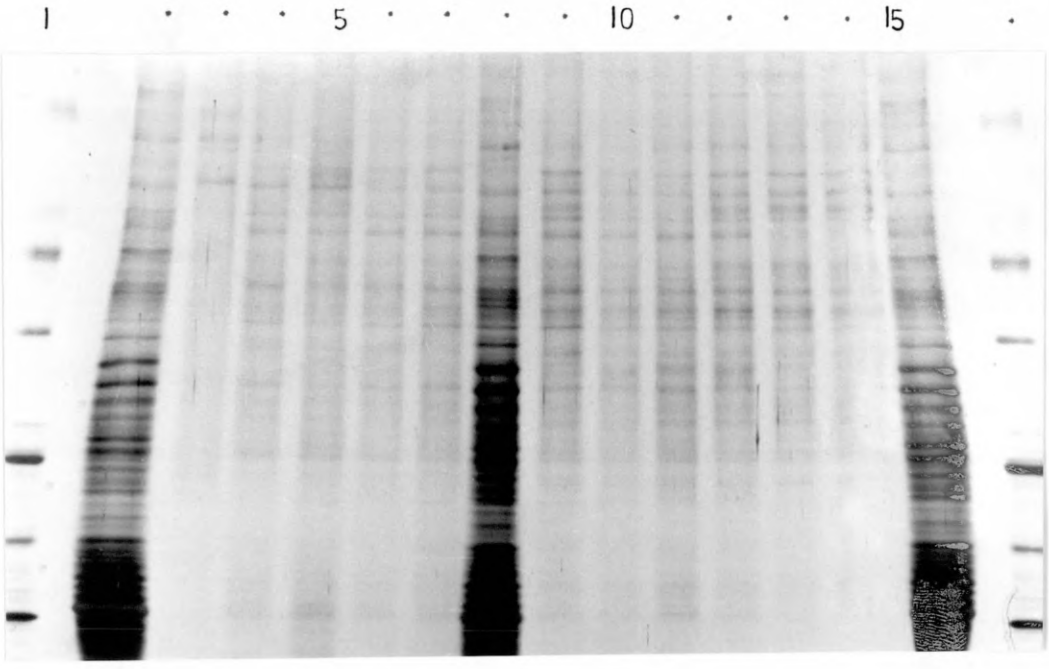
Tracks 2 - 15 represent young mycelium of the following isolates of S. lacrymans: BF-072 (2); BF-050 (3); BF-046 (4); BF-044 (5); BF-025 (6); BF-023 (7); BF-018A (8); BF-017B (9); BF-015B (10); BF-07B (11); BF-03A (12); BF-01 (13); CMI 152233 (14) and FPRL 12C (15).

Figure 40: Western blot analysis of young and aged mycelium of wood decay Basidiomycetes.



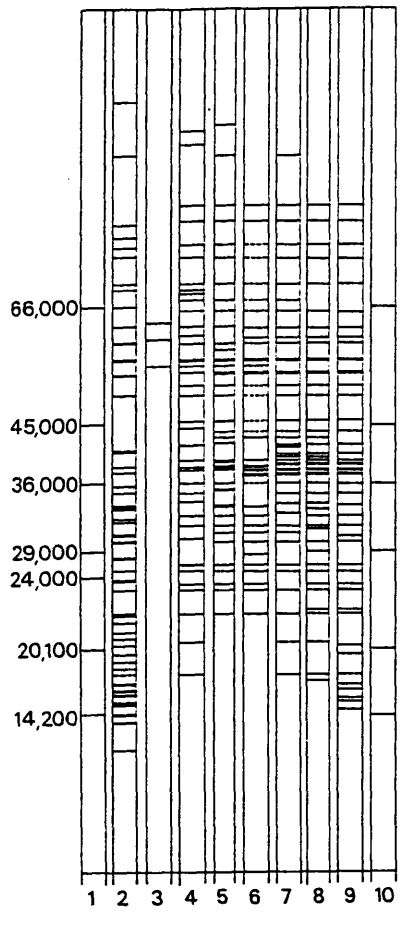
Tracks 1 and 16 represent low molecular weight standard proteins.
Tracks 2, 7 and 15 represent standard mycelium of S. lacrymans FPRL 12C.
Tracks 3 - 6 represent aged mycelium of P. incrassata (3); P. panuoides (4); C. puteana (5) and S. lacrymans FPRL 12C (6).
Tracks 8 - 14 represent young mycelium of S. lacrymans FRPL 12C (8 and 14); C. versicolor (9); P. incrassata (10); P. panuoides (11); F. vaillantii (12) and C. puteana (13).

Figure 41: SDS-PAGE/silver stain analysis of S. lacrymans in pine sapwood.



Tracks 1 and 16 represent low molecular weight standard proteins.
Tracks 2, 8 and 15 represent standard mycelium of S. lacrymans FPRL 12C.
Track 3 represents uninfected wood.
Tracks 4 - 7 and 9 - 14 represent infected pine blocks with the following weight losses: 0% (4); 0.47% (5); 1.02% (6); 4.24% (7); 8.29% (9); 11.03% (10); 15.44% (11); 19.28% (12); 24.51% (13) and 26.73% (14).

Figure 42: Diagrammatic representation of the protein bands associated with extracts of lime infected with S. lacrymans FPRL 12C.



Tracks 1 and 10 represent low molecular weight standard proteins.
Track 2 represents standard mycelium of S. lacrymans FPRL 12C.
Track 3 represents uninfected lime sapwood.
Tracks 4 - 9 represent infected wood with the following weight losses: 0% (4); 0.54% (5); 23.27% (6); 30.46% (7); 54.91% (8) and 61.99% (9).

41, track 8 with 7 and 9. Even at very early stages of infection, i.e. before measurable weight loss (track 4 in both figures) new protein bands could be seen from extracted blocks although some bands apparently corresponded with the standard FPRL 12C profile.

The banding pattern changed during the decay period although there were numerous similarities between the patterns produced at the different decay periods, e.g. as illustrated in Figure 42, the proteins at approximately 36 kDa, 45 kDa and 66 kDa. The protein profiles of S. lacrymans in progressively decayed pine and lime sapwood were also compared with young and aged mycelium. The protein similarity indices for wood grown S. lacrymans FPRL 12C are shown in Table 17 which indicates, firstly, that with similarity indices to standard mycelium of 40.7 - 53.0 (pine) and 34.5 - 37.9 (lime) S. lacrymans FPRL 12C in wood is very different from standard mycelium; and, secondly, that in pine and lime sapwood at low weight losses S. lacrymans FPRL 12C is more like young mycelium than either standard or aged mycelium; thirdly, the organism in wood of higher weight losses is more similar to aged mycelium than to young mycelium and this is also suggested by Table 18 which indicates a change in nature of S. lacrymans as decay of pine and lime progresses.

Confirmation of protein analysis was sought by western blot analysis of pine blocks with weight losses between 0.0 and 26.73% (Figure 43). Undecayed wood showed very limited cross reactivity (track 14) and decayed wood showed bands (tracks 3 - 8 and 10 - 13). The antigenic profiles of material grown on wood or in liquid culture

Table 17: Protein similarity indices of S. lacrymans FPRL 12C in pine and lime sapwood with reference to standard, young and aged mycelium of S. lacrymans FPRL 12C.

		Percentage similarity to		
	% weight loss	Standard	Young	Aged
Pine	0.0	45.9	76.5	47.8
	1.02	47.6	61.9	46.4
	4.24	42.9	67.8	47.2
	11.03	52.6	70.9	46.8
	19.28	53.0	76.3 (4)	56.7
	26.73	52.4	64.7	61.3
	39.84	40.7	59.2 (4)	65.2
	42.5	41.8	53.6 (4)	63.0
Lime	0.0	37.8	71.2 (4)	44.5
	0.54	35.1	65.6	45.7
	23.27	37.2	64.3 (4)	42.4
	30.46	34.5	58.9	57.6
	54.91	35.1	56.1	65.8
	61.99	37.9	57.1 (4)	61.3

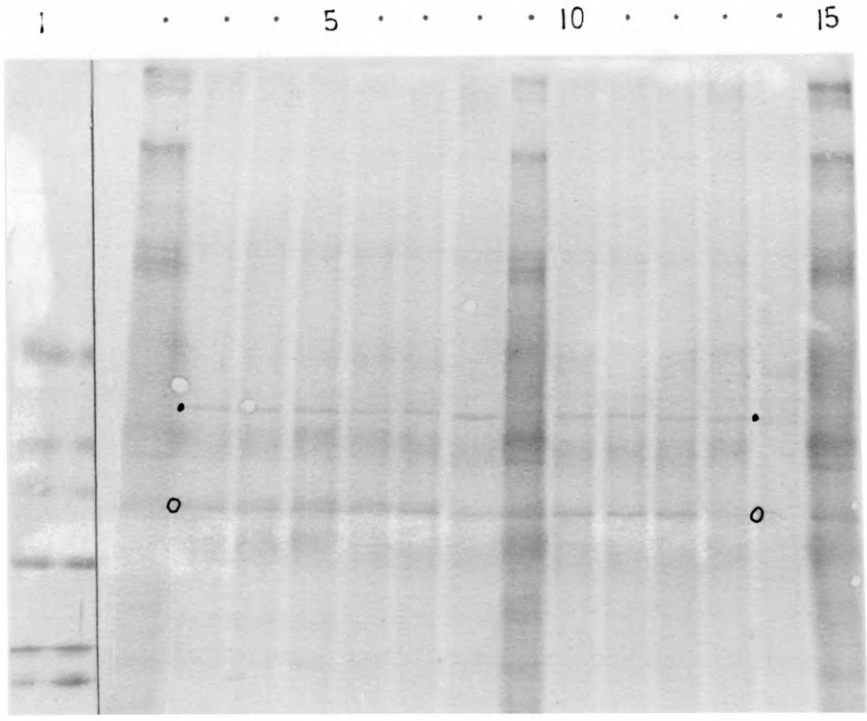
All values were the mean of 2 observations unless otherwise stated in parenthesis ( ).

Table 18: Protein similarity index of S. lacrymans infected pine blocks with reference to 0% weight loss block.

	% weight loss	Percentage similarity
Pine	0.47	85.7
	1.02	85.7
	4.24	80.9
	8.29	78.9
	11.03	78.9
	15.44	71.4
	19.28	71.4
	24.51	71.4
	26.73	71.4
	39.84	65.8
	42.50	63.4
Lime	0.54	82.1
	23.27	84.5
	30.46	76.8
	54.91	70.7
	61.99	62.8

All values were the mean of 2 observations.

Figure 43: Analysis of antigenic proteins of S. lacrymans FPRL 12C in infected pine sapwood.



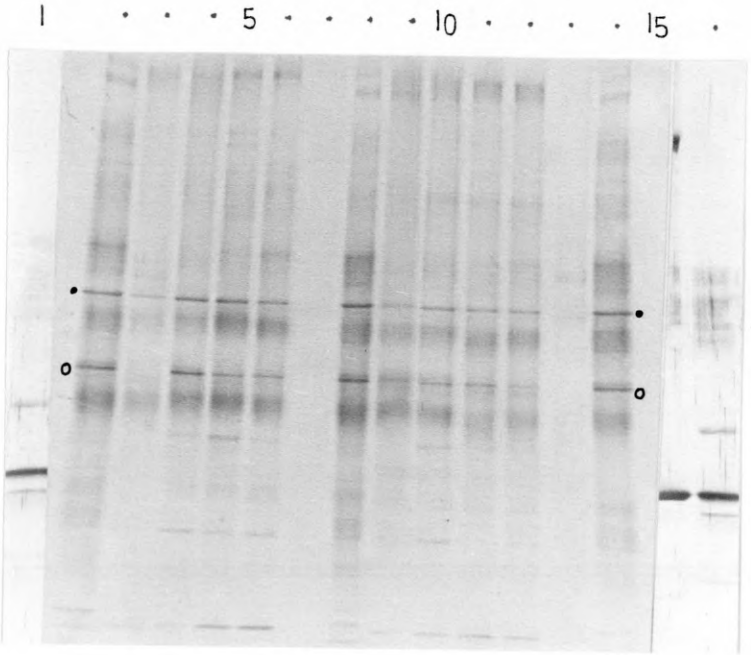
Track 1 represents low molecular weight standard proteins.
Tracks 2, 9 and 15 represent extracts of FPRL 12C standard mycelium.
A sample of uninfected wood is shown in track 14.
Tracks 3 - 8 and 10 - 13 represent extracts from infected wood blocks with the following weight losses: 26.73% (3); 24.51 (4); 19.28% (5); 15.44% (6); 11.03% (7); 8.29% (8); 4.24% (10); 1.02% (11); 0.47% (12) and 0.0% (13).

. and o represent the major antigens, of molecular weights 57 kDa and 43 kDa respectively, found in infected wood.

were rather different (tracks 8 and 9), e.g. the two major diffuse antigens which were present in the standard mycelium were less prominent in the wood material. However, two other antigens which were minor components of the liquid culture extracts, featured as the major antigens of the wood extracts. Comparison of the results shown in Figures 31 and 43 indicates that the antigenic profiles found in young mycelium closely resembled those detected in extracts of pine sapwood grown mycelium. Confirmation of this observation is shown in Figure 44 which indicates that the major antigens found in young mycelium of S. lacrymans FPRL 12C (track 8) and in infected pine and lime sapwood (tracks 10 and 4 respectively) are similar in molecular weight and, possibly, in identity. This is particularly apparent in blocks with weight losses of <27%, e.g. Figure 43, tracks 10 - 13 and 3 - 8; and Figure 44, tracks 4, 5, 6 (lime) and 10. 11, 12 (pine). Blocks of higher weight loss (Figure 44, tracks 3 and 9) indicate a trend towards loss of the major antigens of young mycelium.

Different substrates may also be associated with different profiles. When infected wood blocks were compared either with the infected wood block showing no weight loss (Table 18) or with each other (Table 19) a comparison of the resultant protein similarity indices with Table 17 confirmed that S. lacrymans FPRL 12C grown in wood has a markedly different protein profile to that of the organism grown in artificial media.

Figure 44: The antigenic profiles of young mycelium of S. lacrymans FPRL 12C and pine and lime sapwood infected with S. lacrymans FPRL 12C.



Tracks 1, 15 and 16 represent molecular weight standard proteins.
Tracks 2, 8 and 14 represent young mycelium of S. lacrymans FPRL 12C.
Tracks 7 and 13 respectively represent uninfected lime and pine sapwood.
Tracks 3 - 6 represent infected lime sapwood of the following weight losses: 54.91% (3); 23.27% (4); 0.54% (5) and 0.0% (6).
Tracks 9 - 12 represent infected pine sapwood of the following weight losses: 45.2% (9); 19.28% (10); 1.02% (11) and 0.0% (12).

. and o represent antigens of 57 kDa and 43 kDa respectively.

Table 19: Protein similarity index of S. lacrymans FPRL 12C grown in pine or lime sapwood when compared with each other rather than the standard FPRL 12C preparation.

	Comparison between blocks of stated weight loss		Percentage similarity
	Standard	Test	
Pine	0.0	0.47	95.0
	0.47	1.02	94.7
	1.02	4.24	92.0
	4.24	8.29	93.6
	8.29	11.03	90.5
	11.03	15.44	93.2
	15.44	19.28	87.6
	19.28	24.51	83.5
	24.51	26.73	82.1
	26.73	39.84	81.9
	39.84	42.50	84.5
Lime	0.0	0.54	90.5
	0.54	23.27	95.9
	23.27	30.46	70.7
	30.46	54.91	85.7
	54.91	61.99	88.4

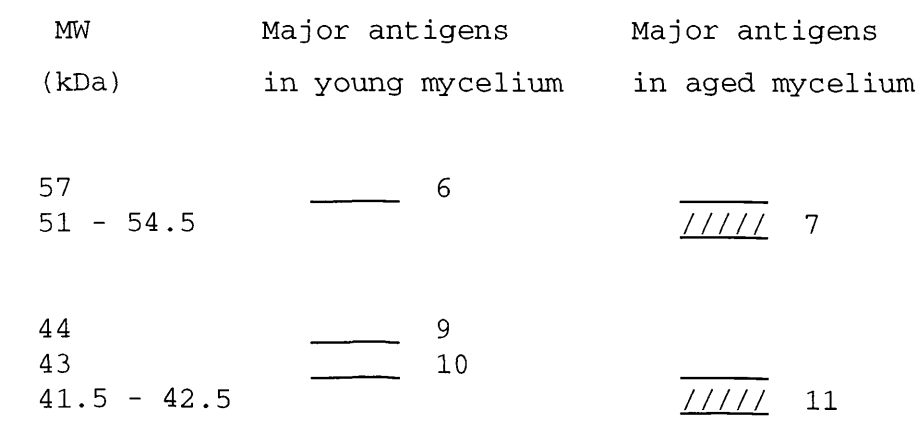
All values are the mean of 2 observations.

7.6 Discussion.

Previous chapters have shown the remarkable consistency of molecular profiles of S. lacrymans. However, these studies also illustrated a set of diverse observations which are further analysed in this chapter, viz the nature of the molecular profile of young and aged mycelium, the identity of isolate BF-050 and the profile of S. lacrymans in wood.

In spite of the difference in the visual appearance of the antigenic profile of young and aged mycelium they have a number of antigens in common with each other. This is not surprising since aged mycelium is differentiated from young mycelium (Hornung & Jennings, 1981). Additionally, since there is a close association in the molecular weights of the major antigens associated with young and aged mycelium, e.g. antigens 6 and 7 (Figure 45), this is perhaps suggestive of a relationship based on derivation from the same initial gene product during differentiation.

Figure 45 : The major antigens of young and aged mycelium.



Two models might be proposed for the derivation of the major antigens of aged mycelium. Firstly, they might be breakdown products of the major antigens in young mycelium produced during experimental preparation. However, this was investigated using *in vitro* incubation techniques which produced no conclusive evidence in favour of this hypothesis. Secondly, the visual appearance of the major antigens might suggest that those of aged mycelium are glycoproteins derived during differentiation either by breakdown and subsequent glycosylation of the major protein antigens of young mycelium or by cleavage of major glycoprotein antigens of young mycelium. This was investigated using peroxidase-linked Con A and indicated that whilst antigens 7 and 11 were glycoproteins so also was antigen 6. This mitigates against the possibility of antigen 6 being the protein precursor of antigen 7 but is consistent with breakdown and reorganisation of antigen 6 into antigen 7.

That antigens 9 and 10 were not stained by Con A merely indicated that these major antigens of young mycelium did not contain terminal α-D-mannosyl or α-D-glucosyl residues. It might be that this result is indicative of the non-glycosylated nature of these antigens and the possibility of their being protein precursors to antigen 11. However, before any conclusions can be drawn about the relationship of antigens 9 and 10 to antigen 11, further investigation of the chemical nature of these antigens is required. Such work might include the use of different lectins which are specific for other sugar residues, e.g. DSA, from *Datura stramonium*, which detects carbohydrate sequences with terminal β-D-N-

acetyl-glucosamine and β-D-galactose (Boehringer Mannheim).

These studies indicate that it is possible that the major antigens in young mycelium are related to those in aged mycelium though further work is required before definite conclusions can be drawn; for example, pulse/chase experiments involving labelling of the major antigens in young mycelium; the development of molecular probes against major antigens in young mycelium, e.g. monoclonal antibodies, to investigate their reaction with the major antigens in aged mycelium; or the inclusion in the growth medium of a glycosylation inhibitor, e.g. tunicamycin, with subsequent investigation of the antigenic profile of aged mycelium.

If molecular profiles are to be used for identification purposes it is essential to have either consistency in banding pattern amongst isolates or knowledge of the range of variation of the species under investigation expressed as a percentage similarity index. Both of these can be achieved only after analysis of a large number of bona fide isolates. Most bona fide isolates of S. lacrymans showed highly consistent profiles to the reference material in 4 tests (SDS-PAGE/silver stain with standard and young mycelium, western blotting and glycoprotein staining). However, whilst isolate BF-050 conformed to the profile for isolate FPRL 12C in 3 types of analysis it produced an anomalous pattern in the basic test (SDS-PAGE/silver stain against standard mycelium). This might lead to misidentification if this were the only test used for identification and could lead to doubt being cast upon the usefulness of

molecular techniques for identification purposes. The extent to which BF-050 is anomalous is difficult to determine since a relatively low number of tests investigating variation have produced one inconsistent result; only if a large number of tests investigated a large number of isolates which produced consistent profiles with the exception of isolate BF-050 in SDS-PAGE/silver staining could the isolate be said to be truly anomalous. An understanding of the range in the variation in S. lacrymans may have been misled by the relatively small number (19) of isolates tested. However, in a smaller study (5 isolates) on C. puteana (McDowell, 1992) and a larger study (30 isolates) on H. annosum (Galbraith, 1992), SDS-PAGE/silver staining illustrated a greater degree of variation from the reference isolate for most organisms within each species. Limited studies have also been done on other Basidiomycetes, e.g. G. trabeum (SDS-PAGE/silver staining) (Hainey, 1992) and L. lepideus (western blotting) (Glancy, 1990), and indicate that variation is to be similarily expected in these species. Thus it may be that S. lacrymans is unusual amongst Basidiomycetes in having a high degree of conformity of profile amongst its isolates. This may be indicative of a relatively short evolutionary history related to a comparatively recent colonisation of building timber.

Results for isolate BF-050 indicate that it is a S. lacrymans isolate but this conclusion was only reached after several tests. Thus this emphasises that the tests used in this thesis do not offer a definitive identification system for anomalous isolates when these tests are used singly. Differences noted between the

SDS-PAGE/silver stain profiles of standard mycelium of BF-050 and FPRL 12C must be attributable to differences in structure or function of the portion of the mycelium outwith the young mycelial area, i.e. older mycelium of BF-050. These differences may be related to an aspect of the environment of this Australian isolate which is shared by neither the two other Australian isolates, BF-046 and BF-049, nor all other isolates. The other anomalous isolate, BF-015B, appeared to be confirmed as a different species to S. lacrymans, but diagnosis as S. himantioides might be confirmed by comparison of young mycelium of the two organisms.

The antigenic profiles in wood were found to resemble either young or aged mycelium depending on the weight loss of the wood. This finding was confirmed by the protein similarity indices. Such profiles could be usefully exploited in studies on the viability and decay potential of S. lacrymans and could enable the colonisation of building timbers by S. lacrymans to be monitored. Determination of viable material is of prime importance commercially and the antigens associated only with young mycelium might be indicative of viability. More work requires to be done to determine the relationship between antigenic profile and organism viability in wood but immunological probes developed against the major antigens of either young or aged mycelium could have the potential to differentiate between viable and non-viable S. lacrymans.

Benhamou et al. (1985), who produced monoclonal antibodies which were used in immunocytochemical techniques to detect the Dutch elm disease pathogen

Ophiostoma ulmi within infected wood samples, were the first to use immunological methods to detect fungi within wood. More recently, studies have demonstrated that immunological probes based on polyclonal antisera can detect Basidiomycetes in wood, e.g. L. lepideus and C. versicolor (Palfreyman et al., 1987). Western blotting has detected C. puteana (McDowell, 1992) and H. annosum (Galbraith, 1992) in decayed wood blocks. The present study has demonstrated that S. lacrymans can similarly be detected in wood. This has potential for the identification in situ of the causative organism of wood decay. However, the differences in both protein and antigenic profiles between the organism grown in liquid culture and the organism cultured in wood pose problems for this identification since it is important to have standardised profiles in order to be able to identify organisms using the present methods. However, the present antiserum illustrates a high degree of antigenic similarity between S. lacrymans grown on different media. This points to potential for the development of immunological detection system since probes, e.g. monoclonal antibodies, raised against particular epitopes are likely to react with the organism grown on a wide variety of substrates which could be particularly useful in the detection of the organism in field material. This has already been investigated (Glancy, personal communication) and results indicate that monoclonal antibodies could be used in simpler systems than western blotting, e.g. ELISA, immunocytochemical techniques or dot immunobinding assays. To confirm the existence of these common epitopes in different morphological field forms the work described in chapter 8 was undertaken.

CHAPTER 8. ANALYSIS OF *S. lacrymans* FIELD SAMPLES.

8.1 General introduction.

Accurate diagnosis of *S. lacrymans* infection in the field is important so that appropriate remedial treatment can be initiated speedily. This thesis has demonstrated that, if allied with the use of similarity indices, SDS-PAGE/silver staining and antigenic profiles can allow objective differentiation between *S. lacrymans* and other wood decay species of economic importance. However, so far the reported studies have been largely limited to isolated material and problems have already been raised in the potential use of molecular profiles for identification of non-isolated material. Firstly, SDS-PAGE/silver stain analysis of non-isolated *S. lacrymans* (see Chapter 5 (5.2.2)) has revealed differences between laboratory cultured mycelium and a few samples of field mycelium, basidiocarp and strand; and between samples within one morphological type, basidiocarp. Secondly, antigen expression in *S. lacrymans* was affected by the morphological growth phase of laboratory mycelium (see Chapter 6 (6.4)). It is, therefore, unlikely that molecular methods could be applicable to identification of non-isolated *S. lacrymans* until the full extent of the variability of protein and antigenic profiles of different morphological forms of *S. lacrymans* grown in the field is understood.

The specific objectives of work in this chapter were

1. To investigate variation in SDS-PAGE/silver stain profiles of *S. lacrymans* in field mycelium, basidiocarp, strand and infected wood.

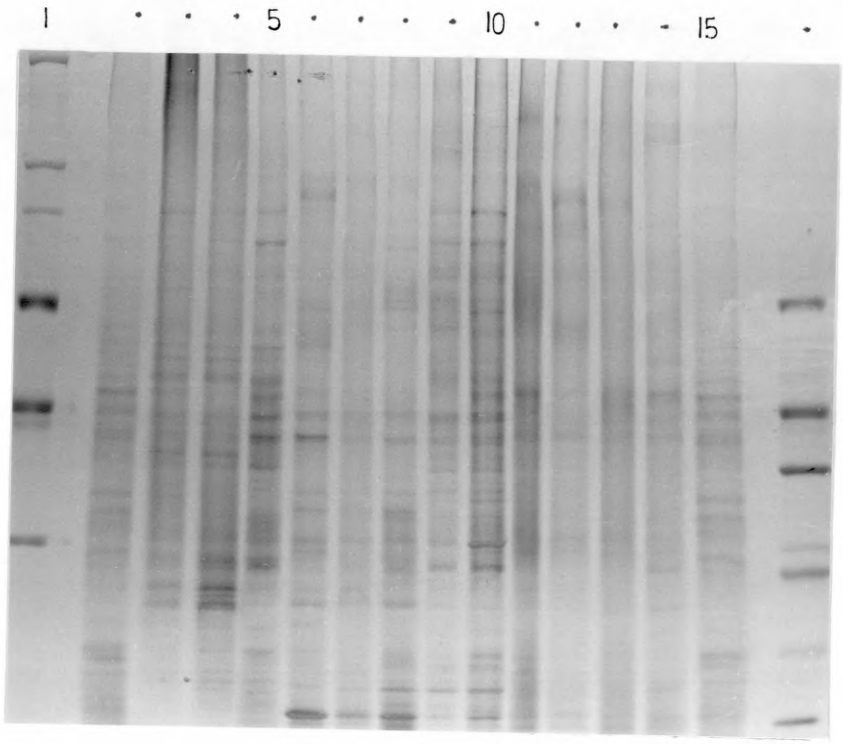
2. To investigate variation in the antigenic profile of field material of S. lacrymans.
3. To attempt to delineate a molecular profile which is unique to each morphological form.
4. To detect any antigens which are associated with certain stages of each growth phase.

8.2 SDS-PAGE/silver stain analysis of field material of S. lacrymans.

Analysis of mycelium, basidiocarp, strand and infected wood was undertaken in order to compare the protein profiles of different morphological forms of field S. lacrymans (Figure 46). The results indicate, firstly, the paucity of proteins in strand (tracks 11 and 12) and infected wood (tracks 13 and 14); secondly, the similarity in pattern between field mycelium (tracks 6 - 8) and basidiocarp (tracks 9 and 10); and, finally, the common and different bands in a comparison between laboratory material (tracks 2 - 5) and field mycelium and basidiocarp (tracks 6 - 10).

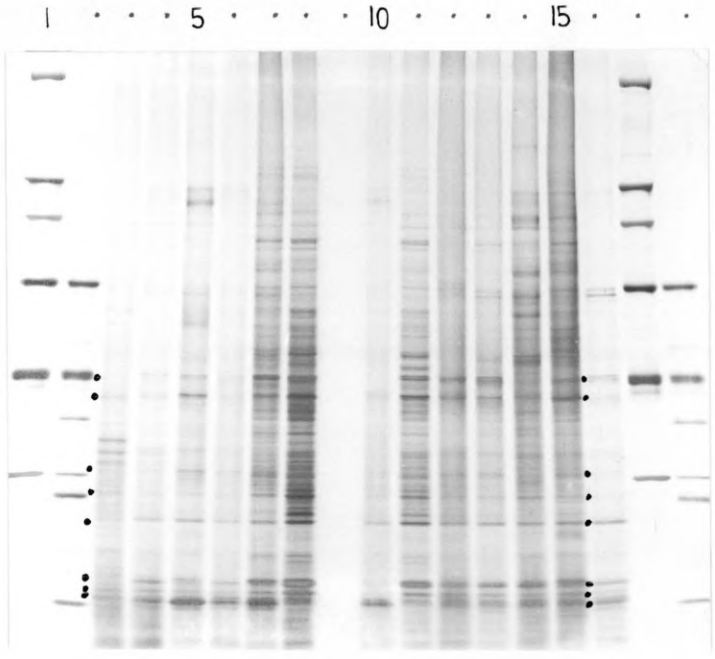
Analysis of the variation in profile within a morphological form was undertaken (Figure 47) and indicated, firstly, that field mycelial samples illustrate a range in the numbers of proteins detected, e.g. compare those with >55 bands, tracks 7, 8 and 11, with those which have <20 bands, tracks 6, 9, 10 and 16. Additionally, there are common proteins in most mycelial samples, e.g. those at 45 kDa, 42 kDa, 30 kDa, 24.5 kDa and those between 14.5 - 17.5 kDa; and some samples, e.g. tracks 7, 8 and 11, have great similarity to

Figure 46: SDS-PAGE/silver stain analysis of field material of S. lacrymans.



Tracks 1 and 16 respectively represent high and low molecular weight standard proteins.
Tracks 2 and 15 represent standard mycelium of S. lacrymans FPRL 12C.
Tracks 3 and 4 respectively represent S. lacrymans FPRL 12C in pine and lime sapwood of 19.28% and 23.27% weight loss.
Track 5 represents young mycelium of S. lacrymans FPRL 12C.
Tracks 6 - 8 represent field mycelium of S. lacrymans.
Tracks 9 and 10 represent field basidiocarp of S. lacrymans.
Tracks 11 and 12 represent field strand of S. lacrymans.
Tracks 13 and 14 represent wood infected with S. lacrymans.

Figure 47: SDS-PAGE/silver stain analysis of field mycelium of S. lacrymans.



Tracks 1 and 17 represent high molecular weight standard proteins.
Tracks 2 and 18 represent low molecular weight standard proteins.
Track 3 represents S. lacrymans FPRL 12C standard mycelium.
Tracks 4 - 16 represent mycelium from site A (tracks 4, 7 - 16); site C (5) and site D (6).
Material diagnosed on collection as 'fresh' is represented in tracks 4 - 8 and 11 - 15; and as 'desiccated' in tracks 6, 9, 10 and 16.

. represents common proteins.

standard mycelium of S. lacrymans FPRL 12C (track 3) and to each other. Analysis of basidiocarp samples (not shown) produced similar results. The protein similarity indices for field morphological forms of S. lacrymans (Table 20) emphasise the similarity of certain samples of field mycelium and basidiocarp to standard mycelium of S. lacrymans. Table 20 also indicates that similarity indices are unsuitable for comparison of all strand and infected wood samples, and some mycelial and basidiocarp samples.

8.3 Western blot analysis of field material of S. lacrymans.

The antigenic profiles of some of the field samples are shown in Figures 48 (mycelium), 49 (basidiocarp), 50 (strand) and 51 (wood). Whilst there are many differences between samples there are also some obvious similarities between standard mycelium of S. lacrymans FPRL 12C and the different morphological forms, e.g. antigens 7 and 11 are probably represented in some samples of all morphological forms. These impressions were confirmed by Figure 52 which compares field mycelium (tracks 6 - 8), basidiocarp (tracks 9 and 10) and strand (tracks 11 - 13) with standard mycelium of S. lacrymans FPRL 12C (tracks 1 and 15). Figure 52 also indicates that the different morphological forms have no greater resemblance to either young and aged mycelium of S. lacrymans FPRL 12C (tracks 5 and 14 respectively) or to the organism in pine and lime sapwood (tracks 3 and 4 respectively).

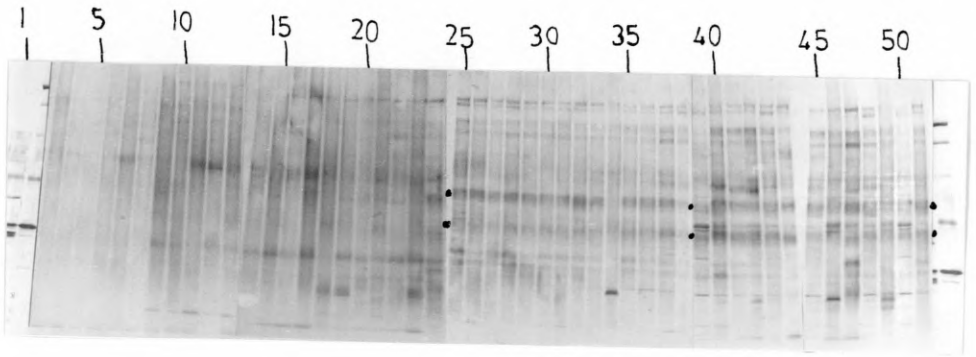
Table 20: Protein similarity indices for S. lacrymans field morphological forms with reference to S. lacrymans FPRL 12C standard mycelium.

Morphological form of field S. lacrymans	Similarity index	Number of samples analysed by SDS-PAGE
Mycelium	55 - 95 (26)	49
Basidiocarp	66 - 92 (6)	29
Strand	*	9
Wood	*	20

* indicates too few molecular species for analysis.

( ) Indicates number of samples in range.

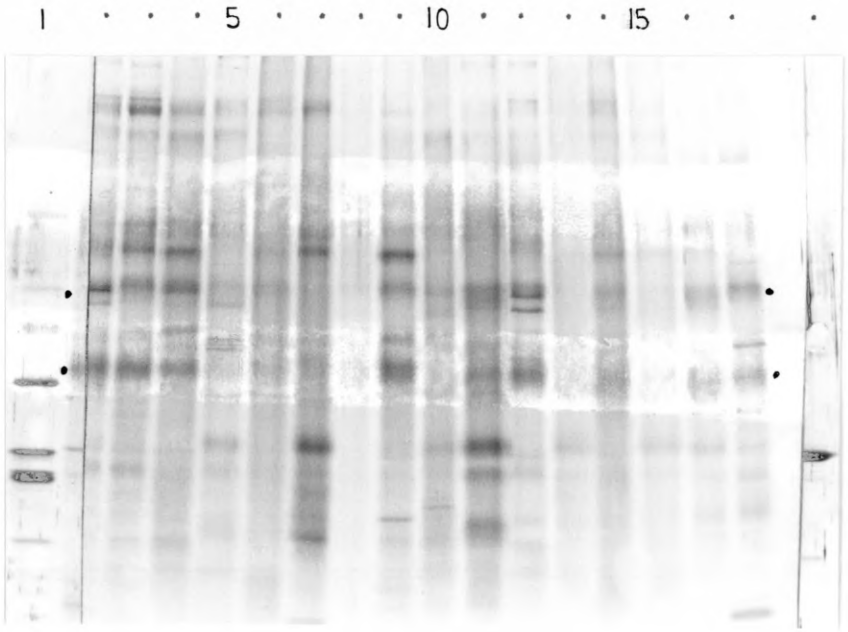
Figure 48: Western blot analysis of field mycelial samples of S. lacrymans.



Tracks 1, 2 and 52 represent molecular weight standard proteins.
Track 51 represents standard mycelium of S. lacrymans FPRL 12C.
Tracks 3 - 50 represent field samples from sites A - E.
Site A - tracks 3 - 14, 25 - 47 and 50.
Site B - tracks 17 - 21 and 23.
Site C - tracks 48 and 49.
Site D - tracks 16 and 22.
Site E - tracks 15 and 24.

. represents antigens 7 and 11.

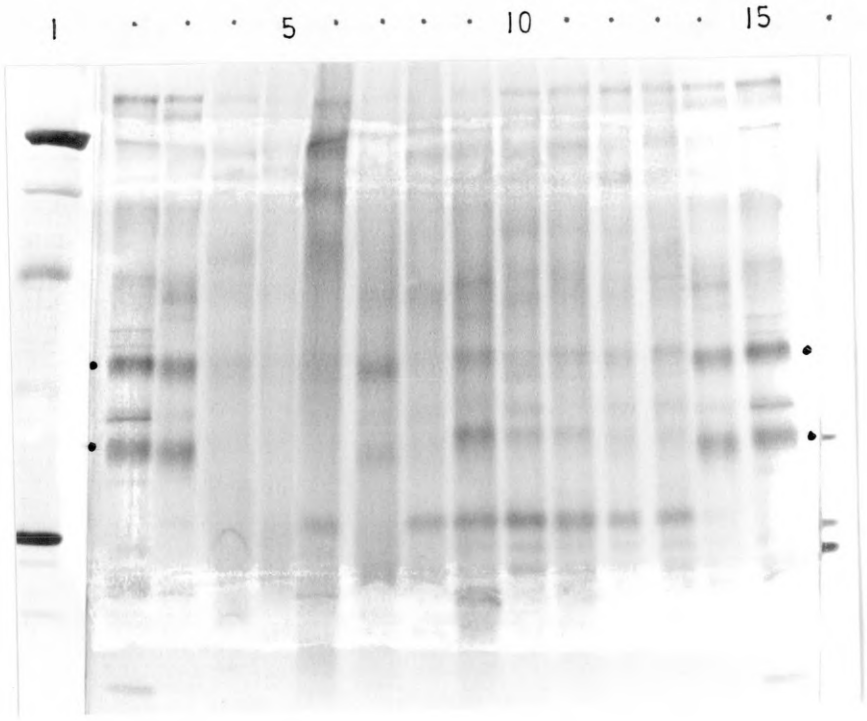
Figure 49: Analysis of the antigenic profile of basidiocarp of S. lacrymans collected from three different sites in Scotland.



Tracks 1 and 18 represent low molecular weight standard proteins.
Track 17 represents standard mycelium of S. lacrymans FPRL 12C.
Track 16 represents aged mycelium of S. lacrymans FPRL 12C.
Tracks 2 - 15 represent field basidiocarp of S. lacrymans from 3 sites: track 2 from site C, tracks 3 - 9 from site B and tracks 10 - 15 from site A.

. represents antigens 7 and 11.

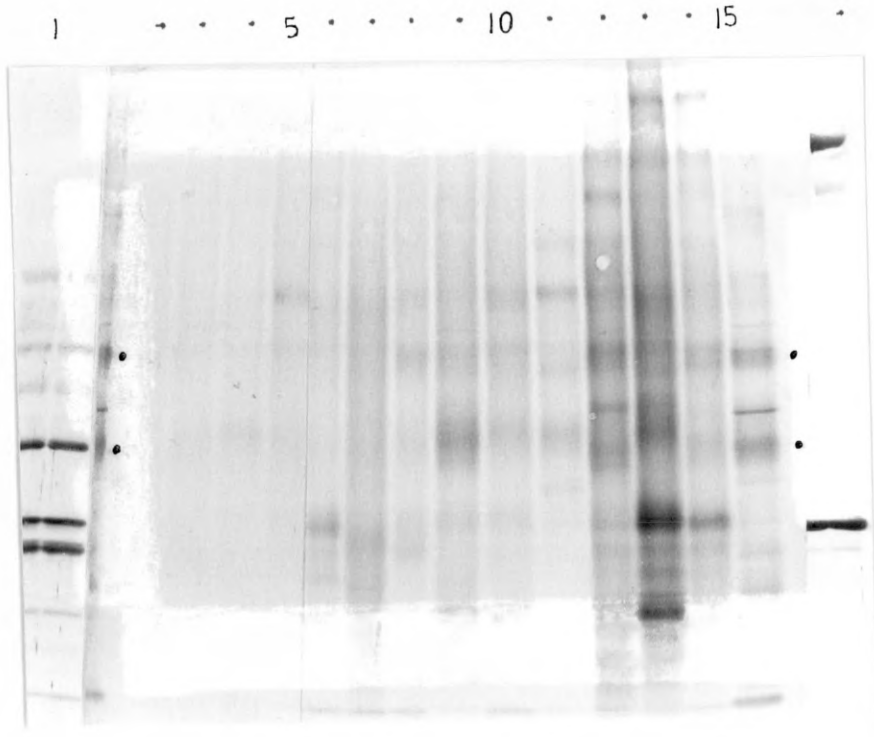
Figure 50: Analysis of the antigenic profile of S. lacrymans strand collected from sites in Scotland and Germany.



Tracks 1 and 16 represent molecular weight standard proteins.
Tracks 2 and 15 represent standard mycelium of S. lacrymans FPRL 12C.
Tracks 3 and 14 represent aged mycelium of S. lacrymans FPRL 12C.
Tracks 4 - 13 represent S. lacrymans field strand samples. Samples collected in Scotland are those in tracks 4, 5, 7, 12 and 13 (site A) and tracks 10 and 11 (site B). Samples collected from sites in Germany are those in tracks 6 and 8 (site E) and track 9 (site D).

. represents antigens 7 and 11.

Figure 51: Analysis of the antigenic profile of wood infected with S. lacrymans collected from different sites in Scotland and Germany.



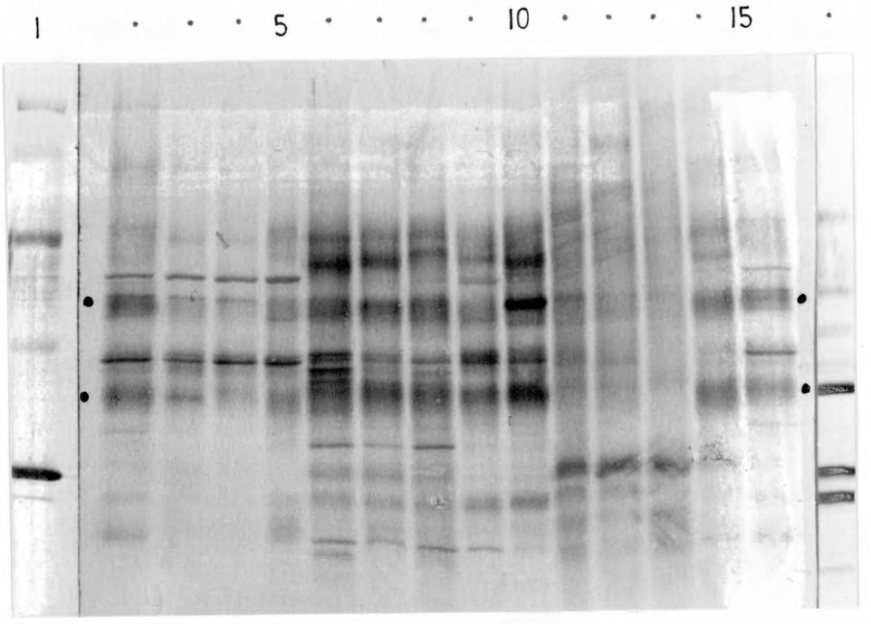
Tracks 1 and 16 represent molecular weight standard proteins.

Track 15 represents standard mycelium of S. lacrymans FPRL 12C.

Tracks 2 - 14 represent infected wood collected from field sites. Track 9 from site D in Germany, with all other samples collected in Scotland, viz tracks 2, 5, 7 and 10 from site A; tracks 6, 12 - 14 from site B; track 8 from site C; tracks 3 and 4 from site I; and track 11 from site J.

. represents antigens 7 and 11.

Figure 52: The comparison of the antigenic profiles of field morphological forms of S. lacrymans with laboratory morphological forms of S. lacrymans FPRL 12C.



Tracks 1 and 16 respectively represent high and low molecular weight standard proteins.
Tracks 2 and 15 represent standard mycelium of S. lacrymans FPRL 12C.
Track 3 represents S. lacrymans FPRL 12C in pine sapwood of 19.28% weight loss.
Track 4 represents S. lacrymans FPRL 12C in lime sapwood of 23.27% weight loss.
Track 5 represents young mycelium of S. lacrymans FPRL 12C.
Track 14 represents aged mycelium of S. lacrymans FPRL 12C.
Tracks 6 - 8 represent field mycelium of S. lacrymans from site C (tracks 6 and 7) and site A (track 8).
Tracks 9 and 10 represent field basidiocarp of S. lacrymans from site C.
Tracks 11 - 13 represent field strand of S. lacrymans from site A (tracks 11 and 12) and site B (track 13).

. represents antigens 7 and 11.

In all morphological forms the antigenic species are represented as diffuse bands, with the exception of a few well defined molecular species in some samples of mycelium (Figure 52, tracks 6 - 8) and basidiocarp (Figure 49, tracks 2 and 12). Antigens 6, 9 and 10 are notably absent from field wood which is infected with S. lacrymans, e.g. Figure 51, tracks 13 - 15, and indicates that differences in antigenic nature are to be expected between S. lacrymans grown in wood in the field situation and the same organism when it is cultured in either pine or lime sapwood (Figure 52, tracks 3 and 4).

All samples within a morphological form illustrate a range in the numbers of antigenic species detectable, e.g. compare either Figure 48, tracks 3 - 8 with tracks 39 - 50; or Figure 49, track 8 with track 3. Potential causes of variation in the numbers of antigens detected in a sample include its site of collection, its freshness upon collection or the surface from which it was collected. That the site of collection is not directly related to the number of antigens was demonstrated for each morphological form, e.g. compare field mycelium (Figure 48) from site A (tracks 46 and 47, 28 antigens) and site C (track 42, 28 antigens); or basidiocarp (Figure 49) from site B (tracks 3 and 8).

However, a directly proportional relationship was observed between the freshness of the field sample upon collection for analysis and the number of antigens subsequently detected, e.g. fresh field mycelium (5 samples) displayed an average of 25 antigenic bands per sample in contrast to desiccated mycelium (5 samples) which displayed an average of 4 bands per sample (data

not shown). Additionally, all mycelial samples (Figure 48) which had been diagnosed as fresh (tracks 25 - 50) contained 11 - 28 antigens whereas, with one exception (track 24), all desiccated samples had 0 - 6 antigens (tracks 3 - 23). The pattern of these results was mirrored in comparisons between fresh and desiccated basidiocarp or strand material. That the surface from which the sample was collected is not directly related to the numbers of antigens detected was illustrated by the mycelial samples since fresh and desiccated samples were each collected from plaster, brick, softwood, stone and carpet.

The antigenic percentage similarity of all samples to standard mycelium of S. lacrymans FPRL 12C was calculated and the antigenic similarity indices for the 4 morphological forms are shown in Table 21 which confirms the differences to standard mycelium of S. lacrymans FPRL 12C of most samples of all morphological forms and indicates that variability in basidiocarp is greater than variability in mycelium, strand or wood. No field mycelial samples were as antigenically similar to S. lacrymans FPRL 12C as bona fide mycelial isolates of the organism (Figure 13).

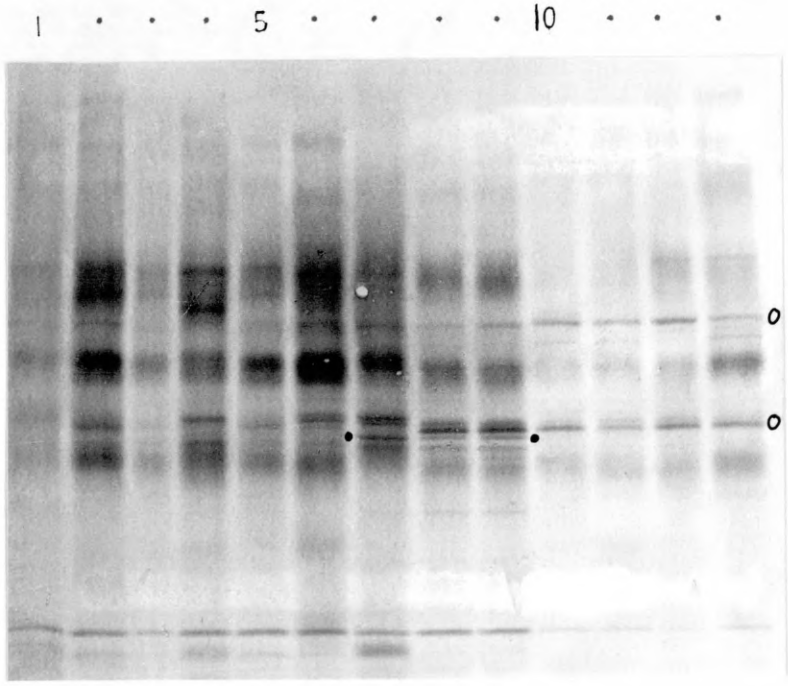
An investigation of the antigenic variability within a morphological form involved analysis of samples from cross sections of fresh mycelial flush and desiccated basidiocarp; no change in antigenic profile was indicated across either morphological form (results not shown). However, changes in representative antigens were observed amongst samples from separate mycelial growths of fresh field mycelium (Figure 53). Young field

Table 21: Antigenic similarity indices of S. lacrymans field samples with reference to standard mycelium of S. lacrymans FPRL 12C.

Morphological form of field S. lacrymans	Similarity index	Total mumber of samples analysed
Mycelium	56 - 79 (26)	49
Basidiocarp	37 - 90 (15)	29
Strand	55 - 85 (6)	9
Wood	56 - 62 (3)	20

( ) indicates number of samples in range.

Figure 53: Analysis of the antigenic variation within fresh field mycelium of S. lacrymans collected from 3 sites in Scotland.



Track 13 represents standard mycelium of S. lacrymans FPRL 12C.
Track 12 represents young mycelium of S. lacrymans FPRL 12C.
Tracks 10 and 11 represent S. lacrymans FPRL 12C grown in lime (23.27% weight loss) and pine (19.28% weight loss) sapwood respectively.
Tracks 1 - 3, 5 - 7 represent composite and tracks 8 and 9 represent young mycelium from site A.
Track 4 represents composite mycelium from site C.

o represents antigens 6, 9 and 10.
. " antigen 10.5.

mycelium (tracks 8 and 9) has similarities to young (track 12), wood grown (tracks 10 and 11) and standard (track 13) S. lacrymans FPRL 12C, e.g. possession of antigens 6, 9 and 10; and lack of antigen 1. However, composite field mycelium (tracks 1 - 7) resembles standard S. lacrymans FPRL 12C mycelium only. An antigen which was novel to mycelium (antigen 10.5) was observed in certain samples of fresh field mycelium, e.g. young mycelium (tracks 8 and 9) and composite mycelium (track 7 only).

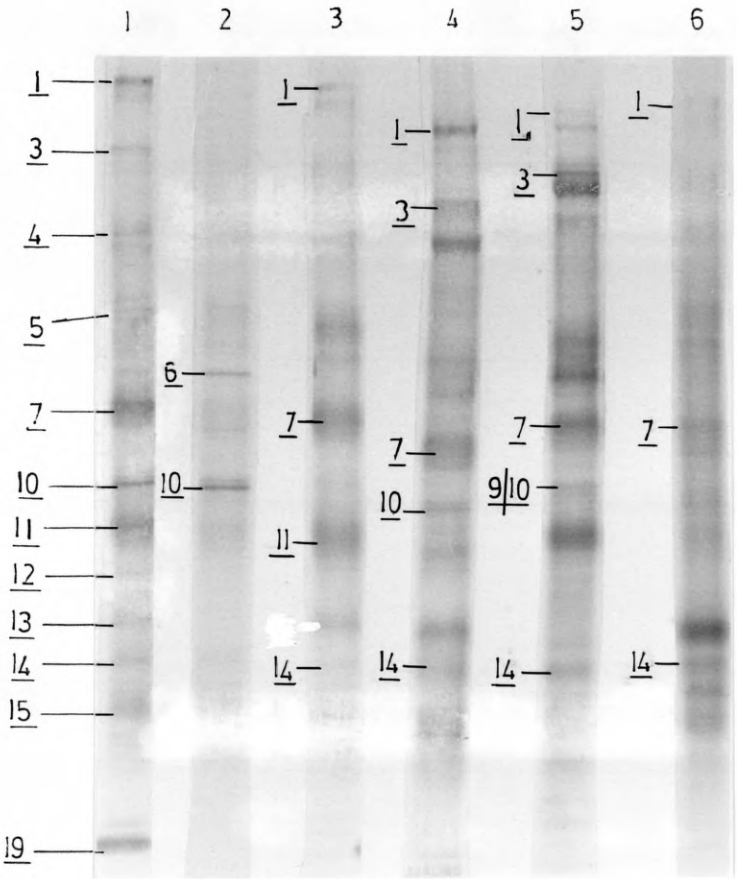
The diagnostic antigenic profile (Chapter 2, 12.2.3) was used for comparison between morphological forms (Table 22). S. lacrymans FPRL 12C standard mycelium had 13 diagnostic antigens (see also Figure 12) whilst field mycelium, basidiocarp and strand had 18, 12 and 10 diagnostic antigens respectively. Representative tracks used to derive the appropriate diagnostic antigenic profile of each morphological form are shown in Figure 54. Only four diagnostic antigens, e.g. 1, 3, 7 and 14, are common to S. lacrymans FPRL 12C standard mycelium and the three morphological forms of the organism (Table 22); and only one other antigen, 3.5, is common to all three field morphological forms. Additionally, an apparent alteration in the nature of two antigenic species is demonstrated by antigens 9 and 10 which can be represented as two sharply defined bands in standard and field mycelium but which feature as one diffuse antigenic species, subsequently referred to as antigen 9/10, in basidiocarp and strand (Figure 54).

An investigation into the distribution and persistence of individual diagnostic antigens in samples was

Table 22: The diagnostic antigenic profile of morphological forms of S. lacrymans.

Antigenic region	Approx. MW (kDa)	Morphological form of S. lacrymans: FPRL 12C standard mycelium	Field mycelium	Field b'iocarp	Field strand
1	180	———	———	———	———
2	160		———	———	
3	116	———	———	———	———
3.25	105		———	———	
3.5	98		———	———	———
4	97.5	———			
4.25	95.5	———			
4.5	78		———		———
5	66	———	———	———	
5.25	64.5	———			
5.5	63		———	———	
5.75	58.5			———	
6	57		———		
7	51 - 54.5	/////	/////	/////	/////
9	44		———		
10	43	———	———	/////	/////
11	41.5 - 42.5	/////	/////	/////	
12	33	———			
13	30 - 31		———		———
14	23.5 - 24.5	———	———	———	———
14.5	23				———
15	22.5	———	———		———
16.5	16		———		
19	12	———	———		
Diagnostic antigens		13	18	12	10

Figure 54: The antigenic profiles of different morphological forms of S. lacrymans.



Track 1 represents standard mycelium of S. lacrymans FPRL 12C.
Track 2 represents young mycelium of S. lacrymans FPRL 12C.
Track 3 represents aged mycelium of S. lacrymans FPRL 12C.
Fresh samples from field collections of S. lacrymans are represented in track 4 (mycelium), track 5 (basidiocarp) and track 6 (strand).

Antigens are represented thus 1, 3 etc.

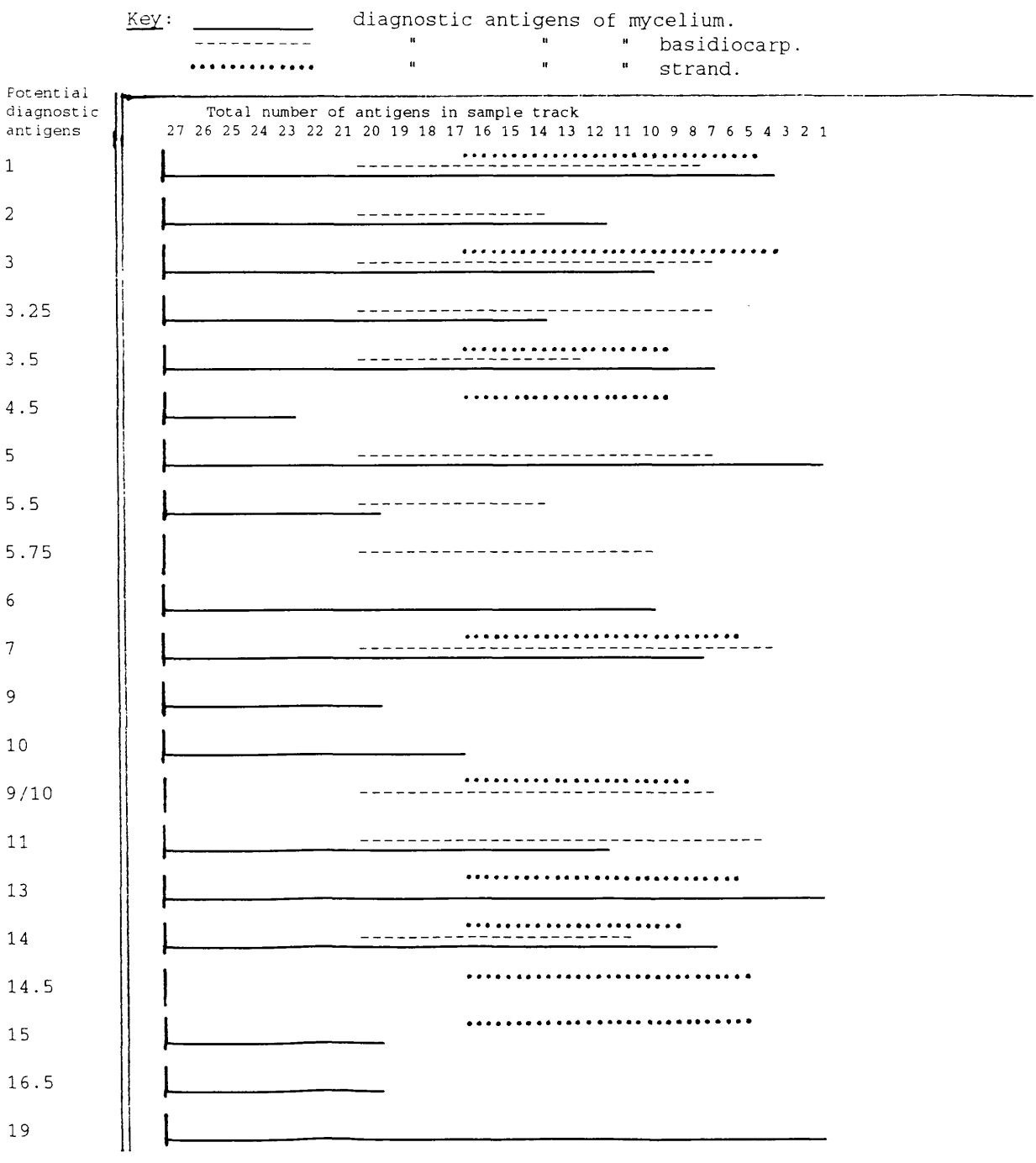
undertaken in order to elucidate the relationship between sample freshness and possession of diagnostic antigens (Table 23). The results show, firstly, that there are certain antigens associated with fresh samples of each morphological type, e.g. basidiocarp is associated with antigens 1, 2, 3, 3.25, 3.5, 5, 5.5, 5.75, 7, 9/10, 11 and 14; secondly, that persistent antigens can be identified by their association with desiccated samples, e.g. desiccated mycelium is associated with antigens 1, 3.5, 5, 7, 13, 14 and 19; and, thirdly, that a pattern exists within the three morphological types in the distribution of the diagnostic antigens between fresh and desiccated material. For example, eleven diagnostic antigens (62%) of field mycelium were found only in fresh samples and six of these were represented only in very fresh (>17 antigens) mycelium. However, only five diagnostic basidiocarp antigens (42%) and two diagnostic strand antigens (22%) were found exclusively in the appropriate fresh material. Thirteen persistent antigens were identified in desiccated samples but their occurrence varies according to morphological type. Persistent antigens comprise 78% of strand diagnostic antigens, 58% of basidiocarp antigens and only 38% of mycelial diagnostic antigens.

Antigens 6, 9 and 10, those major antigens previously associated with young mycelium of S. lacrymans FPRL 12C are represented in very fresh field mycelium. However, it is notable that, whilst antigen 6 is a component molecular species of all fresh samples, antigens 9 and 10 are restricted to samples with >17 antigens and may be indicative of very fresh samples. The major antigenic

Table 23: The possession of specific diagnostic antigens in relation to the total number of antigens of samples of field mycelium, basidiocarp or strand of S. lacrymans.

Samples with >10 antigens were considered fresh, those with <10 were desiccated. Total numbers analysed in each category were:

	Fresh	Desiccated
Mycelium	26	23
Basidiocarp	15	14
Strand	6	3



species of aged mycelium of S. lacrymans FPRL 12C, antigens 7 and 11, are represented in fresh field mycelial samples but only antigen 11 is confined to fresh mycelium. However, these are more persistent antigenic species when represented in basidiocarp or strand.

8.4 Discussion.

This investigation into molecular species in different morphological forms of field collections of S. lacrymans was made by reference to material obtained from controlled growth conditions, viz S. lacrymans FPRL 12C standard mycelium, which itself showed some limited variability due to different morphological content. Analysis of field material revealed further antigenic variation within, and between, each morphological form of the organism and it was demonstrated for all morphological forms that neither substrate nor site of collection had any effect upon antigenic profile.

Further analysis of field material was based upon sample freshness. However, it must be emphasised that an assessment of freshness/desiccation is very subjective since it is based solely on either the appearance of the sample when collected or its association (strand, infected wood) in the field with fresh or desiccated samples of mycelium. Despite this subjectivity it was found that the numbers of antigens in a profile had a direct relationship to the freshness of the sample upon collection. The decline seen in antigen numbers in samples of one morphological form could either be due to

bad storage practices prior to analysis or be indicative of changes associated with differentiation, and concomitant ageing, within that morphological form. The changes observed in the antigenic profile in the progression from fresh to desiccated samples of field mycelium might be related to changes in the nature and function of either the mycelial wall or the cytoplasm which occur as young mycelium ages. Levi & Cowling (1969) noted that fungi whose sole nutrient source is wood may autolyse older parts of the mycelium and re-utilise nitrogenous material in the younger mycelium. In support of this, S. lacrymans is known to break down interconnecting mycelium between strands and recycle the nitrogenous material in the growing hyphae (Coggins, 1976). This process may reduce the number of antigenic molecular species in aged mycelium to below the numbers of those detected in young mycelium.

Antigenic species represented in desiccated samples vary according to morphological form and are possibly related to structural components associated particularly with one morphological form of S. lacrymans. These antigens probably represent molecular species which are highly resistant to degradation and their presence in an antigenic profile in the absence of any other diagnostic antigens could be indicative of senescence and, therefore, non-viability. Identification of antigens associated with senescence might aid in the determination of the ability of field samples of S. lacrymans to continue degradation of timber.

Since the diagnostic antigenic profiles contain species which are exclusive to fresh material, e.g. the defined

antigenic species at 43, 44 and 57 kDa which are associated only with fresh mycelium, it is additionally possible that other antigenic species associated with particular stages of differentiation could be identified within each morphological form of S. lacrymans. The identification of antigens particularly associated with viable developmental stages of S. lacrymans also has the potential to determine the capability of the organism to cause infection. These antigenic species could additionally be used to develop more precise tools for investigation of functions exclusive to viable material and could provide information on the processes associated with morphogenesis of S. lacrymans, i.e. wall differentiation or maturation of cytoplasm.

Since the vegetative mycelial tip is the form of the fungus which explores and exploits the substrate it could be that identification of those antigens particularly associated only with fresh mycelium might be indicative of the morphological phase of the organism capable of causing structural damage to timber. However, whilst the major antigens associated with active growth of young mycelium have been demonstrated in a few mycelial field samples they have not yet been identified in samples of field wood. Alternatively, the observation that senescence can apparently be detected antigenically in field samples suggests that non-viability of the organism in wood might be determined by identification of antigens associated with desiccated samples. However, elucidation of a reliable method of determination of organism status within field wood requires further investigation of the antigenic profiles associated with invasion and decay of wood by S. lacrymans.

Interestingly, the diagnostic antigenic profile of mycelium contains more species than either strand or basidiocarp. This may reflect the nature of the immunogen and/or be indicative of a higher level of physiological activity in the least differentiated morphological form of S. lacrymans. In support of the latter are comparisons between two morphological regions of mycelium, viz young and aged mycelium, where the findings of Pugh & Cawson (1977) confirmed that the enzyme content of young hyphae of Aspergillus and Mucor was greatest at the growing tips and the peripheral enzyme concentration lessened behind the growing point. A comparison between the proportion of persistent antigens in fresh strand and fresh mycelium suggests that strand material, with a higher proportion of ubiquitous persistent antigens, undergoes fewer changes during morphogenesis than mycelium. A similar comparison between the proportion of antigens exclusive to fresh material indicates that fresh mycelium may be physiologically more active than either basidiocarp or strand.

This discussion has so far emphasised the differences in antigenic profiles revealed by the present antiserum in different morphological forms of S. lacrymans and the possible exploitation of these differences. However, the other important aspect of this method of analysis must be that the present antiserum has revealed a considerable degree of similarity between morphological forms of field material, e.g. antigens at 180 kDa, 116 kDa, 98 kDa, the diffuse major antigen between 51 - 54.5

kDa and between 23.5 - 24.5 kDa are ubiquitous to the structure of field grown S. lacrymans.

The common antigenic components may represent similar antigenic species, in which case they are unaffected by differentiation, or they may represent unrelated molecular species which share common epitopes and which may have been derived from mycelial antigens during differentiation. Either way, work using the present non-specific antiserum has indicated that the development of more specific immunological reagents could have potential to determine not only the presence of all morphological forms of S. lacrymans in field material but also to determine whether the sample is fresh, and potentially viable, or desiccated, and of no danger to the structural integrity of the timber in which it is found. However, more reliable information on the similarities and differences between different morphological forms and developmental stages of S. lacrymans requires the use of better discriminatory techniques, which in the context of work in this thesis might involve the development of monoclonal antibodies raised against antigens specific for species or morphological form of S. lacrymans.

CHAPTER 9. GENERAL DISCUSSION.

9.1 Molecular analysis.

S. lacrymans is an extremely successful organism which is superbly adapted to colonise a niche in a potentially harsh environment, i.e. dry building timber. It thrives in ill-lit cavities (Figure 55a) to which it has gained access by exploitation of mistakes in building construction and/or maintenance which allow wood to be wet for long periods prior to remedy of the fault. Once established within a building its physiological nature enables it to invade and decay surrounding dry wood. So successful has been its adaptation to this environment that it is capable of causing tremendous structural damage to wood (Figures 55a and 55b), with devastating effects economically, before its presence is noticed. Obviously the aim should be to detect S. lacrymans at the earliest stage of colonisation of wood, and to determine the extent of colonisation by speedy and non-destructive detection methods. However, traditional methods of detection and survey are relatively crude and have not facilitated the realisation of these aims.

More sophisticated methods of detection have recently become potentially available for the detection of a small biomass of S. lacrymans. Some of these methods are based on changes in the properties of wood as a result of decay, e.g. thermal emission, ultra-sound and Eddy current (Madsen & Adelhoej, 1989), but others are based on the detection of chemicals from the decay organism, e.g. either volatile chemicals or the molecules comprising the organism itself. Volatile chemicals can be detected by dogs (Madsen & Adelhoej, 1989) but there are limitations to the use of dogs (Bech-Andersen, 1991) because it has been found that, firstly, dogs have difficulty differentiating the smell of S. lacrymans from

Figure 55: Serpula lacrymans inside a private house.

Figure 55a: Inside a sub-floor cavity; strands of Serpula lacrymans suspended from rotted floor joists.

Figure 55b: Floor boards rotted by S. lacrymans and a mycelial flush on the carpet.

that of C. puteana and, secondly, they do not react to even serious attacks of other fungi, e.g. F. vaillantii. Other methods based on gas chromatography are currently being investigated for the detection and determination of volatiles from wood decay fungi, e.g. Bjurman & Kristensson (1992) and Esser & Tas (1992). Molecular methods have proved to have potential for detection of basidiomycete mycelium in wood, e.g. immunological studies on P. placenta (Goodell & Jellison, 1986; and Goodell et al., 1988); C. versicolor (Palfreyman et al., 1987) and L. lepideus (Glancy et al., 1989). This project has demonstrated that immunological methods have potential for the detection of S. lacrymans in wood.

A primary aim of this thesis was to use SDS-PAGE/silver staining and western blotting to investigate the protein and antigenic profiles of isolated S. lacrymans grown in controlled conditions, and to examine how these profiles were affected by variation in cultural conditions. This is because there must be an understanding of the molecular nature of the organism when grown in controlled conditions before a meaningful attempt can be made to use similar techniques to detect the same organism in wood. Additionally, if molecular profiles are to be used for detection of S. lacrymans then it is important that different species which are likely to be found in a similar environment have profiles which are different from S. lacrymans. The data reported in this thesis showed conclusively that the molecular methods which were used enabled the delineation of a profile which was unique to isolates of S. lacrymans.

The investigation of molecular profiles of isolates of Basidiomycetes necessitated the development of a relatively speedy and simple method of computation of the similarities between species. The primary objective of this was to defend identification as S. lacrymans, and was used in this thesis to confirm standard mycelium of isolates DIT-101 and DIT-102, and young mycelium of isolate BF-050, as S. lacrymans. However, a development was that either SDS-PAGE/silver staining or western blotting, used in conjunction with an appropriate reference isolate and previously established protein or antigenic similarity indices, could aid in the identification of unknown isolates of other species. This was subsequently demonstrated by the use of SDS-PAGE/silver staining in the identification of unknown isolates as L. sulphureus (McDowell, 1992); G. trabeum (Hainey, personal communication) and H. annosum (Galbraith, personal communication). Western blotting has been similarly used to confirm identity of C. puteana (McDowell, 1992) and, in this thesis, to indicate that isolate BF-015B is probably confirmed as S. himantioides.

It was initially felt that the development of similarity indices might also aid taxonomic studies. However, the methods which employ SDS-PAGE/silver stain for taxonomic studies, e.g. Kersters (1985), are based on computer analysis of the inter-relationships of large numbers of closely related isolates; whereas similarity indices are based on comparison of one organism with an appropriate reference organism. The similarities between the antigenic profiles of S. lacrymans and S. himantioides are probably explained by relationship within a genus, and similarities between these two organisms and C. puteana are possibly explained by taxonomic relationship within a family.

However, it is doubtful if antigenic profiles derived from a non-specific antiserum could contribute to large scale taxonomic studies since other work in this thesis has indicated, firstly, that common antigens detected by such an antiserum are not necessarily indicative of taxonomic closeness; and, secondly, that all genera within a family do not share common antigens. Use of immunological reagents for taxonomic purposes within the Basidiomycetes probably depends upon the development of isolate, species and genus specific monoclonal antibodies. Such probes have been developed in other areas of mycology and were considered to have potential for taxonomic purposes, e.g. Hardham, Suzaki & Perkin (1986) raised monoclonal antibodies against the zoospores and cysts of Phytophthora cinnamoni.

9.2 Detection of field samples.

One drawback to the use of isolates for the identification, and concomitant detection, of S. lacrymans is the time required for the isolation of the infective organism and, for this reason, it would be desirable to detect the organism directly from any morphological form of field material. However, the data showed that SDS-PAGE/silver staining allied with similarity indices was not appropriate for comparison of either many mycelial and basidiocarp samples or all strand and infected wood samples; and the protein similarity indices showed that the variation encountered within the samples of field mycelium and basidiocarp which could be analysed was greater than that amongst the range of S. lacrymans isolates. It is unlikely, therefore, that SDS-PAGE/silver stained profiles will have

universal use for identification of field material as S. lacrymans.

Western blotting identified antigenic species in most field samples, including infected wood but antigenic profiles of field material were initially expected to have even less potential than protein profiles for detection and identification of field S. lacrymans since, firstly, most samples had profiles which showed great variation from isolated mycelium; and, secondly, the antiserum displayed a great deal of crossreactivity with other fungal species. Numerous authors have reported that fungal antisera cross react widely with other Basidiomycetes, e.g. Goodell & Jellison (1986); Glancy (1990); M^{c}Dowell (1992); and Galbraith (personal communication). For some types of detection system there are advantages in having a relatively non-specific antiserum, e.g. in distribution poles it is useful to detect colonisation by a range of Basidiomycetes, since all are capable of degrading the timber. However, exact identification of species is required in the case of S. lacrymans since appropriate remedial treatment depends upon correct identification. Therefore, the way forward to reliable identification of S. lacrymans probably depends upon the more sophisticated molecular technologies currently available, e.g. either the development of monoclonal antibodies against selected fungal molecules or the use of DNA fingerprinting methods.

This thesis has identified antigens which could be used for development of monoclonal antibodies. For example it has shown that, firstly, a particular morphological stage of the organism may be associated with certain antigens, e.g. either the major antigens of young and aged mycelium or the

pattern of antigens uniquely associated with each morphological form of fresh field S. lacrymans; secondly, there are some antigens which are common to laboratory mycelium and fresh field mycelium, strand and basidiocarp; and, thirdly, there are persistent antigens which are associated with desiccated field S. lacrymans. However, the ultimate ideal of differentiation between dead/dormant S. lacrymans and actively growing S. lacrymans might depend upon the development of probes against either the antigens associated with viable S. lacrymans or the enzymes produced only when the organism is actively growing in wood. This would require investigation of the relationship between viability and the antigenic nature of S. lacrymans in field wood and, additionally, determination of the appearance and/or disappearance of specific molecules during the decay process. Probes developed against viable S. lacrymans in wood could additionally aid elucidation of the mechanisms involved in the degradation of wood and could give information on the effectiveness of control/eradication treatments.

Monoclonal antibodies were raised (Glancy, personal communication) against either mycelial extract of standard mycelium or exo-antigens (Dewey, M^{ac}Donald & Phillips, 1989) of S. lacrymans and, whilst the latter method produced only non-specific antibodies, the former method produced three specific antibodies which reacted with a limited number of samples of field mycelium, strand and basidiocarp but not with spores. Whilst these antibodies reacted with infected pine and lime sapwood blocks above a weight loss of 10% they did not react with infected wood samples from the field (Glancy, personal communication). Similar work has resulted in monoclonal antibodies which are specific for isolates of

C. puteana (McDowell, 1992) and H. annosum (Galbraith, personal communication) and which also detect the appropriate organism in wood (both organisms) and field samples (C. puteana only). However, problems have been encountered in developing the use of the monoclonal antibodies for the detection of S. lacrymans (Palfreyman, personal communication). These problems, also reported by Koch (personal communication) in development of a similar diagnostic probe, are chiefly caused by non-specific binding during the ELISA assay. It is probable that the cause is the same (Palfreyman, personal communication) as the problem encountered during the dot immunobinding assay detailed in this thesis, viz reaction of a component of the polyclonal HRP-linked antibody with the antigen, but which was fortuitously overcome by sample preparation prior to electrophoresis. The problem was partially solved by Glancy (personal communication) with the use of alkaline phosphatase-linked antibodies but further advances in the development of a monoclonal antibody based detection system would have to determine the cause of the non-specific binding in order to eliminate it.

9.3 Origins of S. lacrymans.

Though S. lacrymans and S. himantioides are very similar organisms, as evidenced by their presence in the same genus, it has been reported over the years that each occupied different ecological niches. It has been stated that S. lacrymans is confined to buildings whilst S. himantioides is the member of the genus which is found in nature (Cartwright & Findlay, 1958). However, this statement is in disagreement with published literature since S. lacrymans has been

recorded on three occasions from nature (Bagchee, 1954; Harmsen, 1960; and Soukup, 1979) and it is generally accepted that S. himantioides, found in nature on the lower side of fallen conifers, telegraph poles and fence posts (Bech-Andersen, 1991), has been documented in houses (Koch, personal communication). It must be either that both organisms grow in the open and in buildings or that mistakes were made in identification of the organism from the open forest as S. lacrymans with the consequence that only S. himantioides grows in nature and in buildings. It is easy for a non-expert to comprehend how mistakes in identity could occur since the spores and most microscopic characters of S. himantioides and S. lacrymans are similar (Bech-Andersen, 1991). The surface mycelium of S. himantioides is sparser and more membraneous than S. lacrymans and its fruit body does not contain the L-shaped skeletal hyphae which are characteristic of S. lacrymans (Bech-Andersen, 1991) but these are distinctions which would be hard for anyone other than an expert to defend. Indeed, this is implicit in the statement by Bagchee (1954) that critical cultural experiments had yet to confirm the existence of an apparently identical organism in the open forest and local buildings.

If it were proved that S. lacrymans grows in the open forest in the Himalayas it would re-open discussion (Bagchee, 1954) as to its true geographical distribution in nature. It might be that 'wild' S. lacrymans is either widespread in most temperate coniferous forests or grows only in a few regions of the world. In the former case the organism may have escaped detection because environmental pressures, e.g. air movements or temperature variations, restrict its distribution to relatively few places which are hidden from

casual glance and where the economic effects of its action are minimal. If 'wild' S. lacrymans is restricted to a few regions of the world it would raise the question of the mechanism of dispersal to all temperate regions of the world. It could be that either aerial spore dispersal enabled the organism to colonise building timbers as man's activities made them available or incorporation of infected logs into ships during repairs in the country where the organism was indigenous facilitated dispersal worldwide. If spores were responsible for worldwide dispersal of S. lacrymans it can only be a topic of conjecture when the organism first invaded and exploited man's timber constructions. Spores may always have been associated with construction timber in temperate regions and the inevitability of decay following incorporation into a suitable environment was noted as undesirable but had to be accepted prior to identification of the cause of decay and the determination of remedial action.

The European voyages of discovery which began in the early fifteenth century (Times Concise Atlas of World History, 1986) used wooden ships which might have particularly aided the dispersal of infected wood. These voyages opened up a new era in world history after 1500 since they resulted in direct sea contact being established between continents and regions which hitherto had been isolated from each other, e.g. Vasco da Gama reached India in 1499 (Times Concise Atlas of World History, 1986). The return to temperate European countries of ships with infected timbers which were then discarded during repair might have enabled S. lacrymans to spread to uninfected timbers which were subsequently used for building. It must be assumed that S. lacrymans grew in the parts of the construction which provided conditions

similar to those in its natural environment. However, the environmental controls on growth which might be applicable in the wild, e.g. either seasonal and diurnal variations in temperature, humidity and air movements or competitors, would be absent from parts of man's constructions. Within such favourable habitats S. lacrymans would be freed from previous environmental constraints and could demonstrate its full potential for decomposition of timber. The difficulties experienced in the culture of S. lacrymans, which are in marked contrast to its readiness to grow in building timbers, are perhaps indicative of an organism which has become highly adapted to a narrow niche. Such an adaptation might severely limit the distribution of the organism were it not for the fact that the niche is in apparently unlimited supply as an integral part of an essential of man's life.

The use of infected timber or aerial spore dispersal could have initially resulted in the incorporation and subsequent development of both S. lacrymans and S. himantioides in buildings. Nowadays S. himantioides is economically less important in buildings than S. lacrymans but its presence can cause problems after remedial treatment by heat against S. lacrymans (Koch, personal communication) because it is insensitive to temperatures which kill S. lacrymans. The continued growth of S. himantioides after heat treatment, combined with its visual similarity to S. lacrymans, can result in heat treatment against S. lacrymans being deemed ineffective. S. himantioides is the smaller organism (Cartwright & Findlay, 1958) but it must be questioned why S. lacrymans is now more successful in an environment which might initially have provided ideal growth conditions for both organisms. It is possible that this is related to the

range of temperature within which each species grows and which would determine the parts of a building which would be colonised by each species. The growth range for S. lacrymans of 3°C - 25°C, with an optimum of 22°C (Cartwright & Findlay, 1958), would limit the organism to parts of a building which were cooler than those open to colonisation by S. himantioides which, with a growth optimum of 28°C (Cartwright & Findlay, 1958), has a requirement for greater warmth to develop its full potential for growth. It is expensive to heat buildings; therefore, the organism which can grow at low temperatures is more likely to colonise a building provided that all other requirements for growth, e.g. moisture, are satisfied. Even in modern centrally heated buildings there may be cooler parts which inadvertently become damp and if they are inaccessible to regular inspection, e.g. sub-floor cavities and wall spaces, it is likely that S. lacrymans could develop undetected. S. himantioides may have fewer opportunities to develop in modern buildings since the warmer parts of a building are more likely to be dry and easily visible to inspection.

If S. lacrymans is restricted to timber constructions (Cartwright & Findlay, 1958) the implication must be that S. lacrymans does not have a very long evolutionary history which might be confirmed by the conformity of profile between most S. lacrymans isolates. Similar conformity was noted between isolates of aggressive or non-aggressive strains of Ceratocystis ulmi after SDS-PAGE analysis (Jeng & Hubbes, 1983); and Scott (1991) found little or no difference between electrophoretic patterns of Phytophthora megasperma from Europe and America. However, this conformity is at odds with the expection of variation between individuals at the species and sub-species level (Foster,

1949) which was illustrated by the more variable profiles of some Basidiomycete isolates, e.g. C. puteana (McDowell, 1992), P. placenta (Jellison, personal communication) and H. annosum (Galbraith, personal communication); isolates of Phytophthora species (Hall, Zentmeyer & Erwin, 1969); and strains of Aspergillus fumigatus (Hearn et al., 1990) which perhaps reflect a longer evolutionary history. However, it may be that a larger scale analysis of S. lacrymans isolates from around the world would reveal less conformity in profile. If S. lacrymans is restricted to buildings the origin of the organism would have to be questioned: S. himantioides might be the precursor 'wild' organism which was inadvertently incorporated into the first timber constructions but which developed into S. lacrymans in tandem with the provision of increasingly favourable growth conditions, e.g. stability of temperature, RH and air movements, in buildings. The differences shown by the protein similarity indices of standard mycelium of S. lacrymans and isolate BF-050, and also of S. himantioides and isolate BF-015B, have been ascribed previously in this thesis to possible speciation so it is equally possible that this process led to the divergence of S. lacrymans from S. himantioides.

In summary, this project has shown that molecular techniques, in particular SDS-PAGE/silver staining and western blotting, can be successfully used to delineate a molecular profile unique for S. lacrymans against which the effects of substrate and other laboratory growth conditions and growth in the field could be assessed. In addition, a method was established for comparison of molecular profiles and the use of molecular profiles for identification and taxonomic purposes was investigated. Further development of

various areas, e.g. more precise antigenic probes, might allow the development of a routine diagnostic test for S. lacrymans and its determination in field conditions, especially wood. Because they are more objective than visual comparisons the molecular analytical methods allied with similarity indices used in this thesis have the potential to eliminate discrepancies in description of the habitat of S. lacrymans. All investigative techniques require samples to analyse and the most detailed descriptions of the source of S. lacrymans in the open forest are given in Bagchee (1954). Problems would inevitably be associated with the location of a fungus which was found in the 1950's on logs in felling coupes located NW of Simla (Punjab, India) in the inner Himalayas at altitudes between 2400 - 3155m. However an expedition in the summer of 1992 to the Himalayas succeeded in finding an organism resembling S. lacrymans in an area which was eighteen miles from human habitation (Singh, personal communication).

REFERENCES.

ABOU-HEILAH, A.N. & HUTCHINSON, S.A. (1978). Factors contributing to differences between wood decaying abilities of strains of Serpula lacrimans. Trans. Br. Mycol. Soc. 70, 302 - 304.

AINSWORTH, G.C. (1963). Ainsworth and Bisby's Dictionary of the Fungi. Commonwealth Mycological Institute, Kew, Surrey.

ALBION, R.G. (1926). Forests and Sea Power. U.S.A., Cambridge.

ANDER, P. & ERIKSSON, K.-E. (1978). Lignin degradation and utilisation by microorganisms. In : Progress in Industrial Microbiology (ed M.J. Bull), vol. 14, pps 1 -58. Amsterdam, Holland ; Elsevier Publishing Company.

AYERS, A.R. & ERIKSSON, K.-E. (1982). Cellobiose oxidase from Sporotrichum pulverulentum. Methods in Enzymology 89, 129 - 135.

BACON, F. (1612). Essay no. 14 'of nobility'. In : The essaies of Sr Francis Bacon Knight the Kings Solliciter Generall. London, U.K. ; Iohn Beale.

BAGCHEE, K. (1954). Merulius lacrymans (Wulf) Fr in India. Sydowia 8, 80 - 85.

BARTNICKI-GARCIA, S. (1968). Cell wall chemistry, morphogenesis, and taxonomy of Fungi. Ann. Rev. Microbiol. 22, 87 - 108.

BATSON, R. (1794). Trans. Soc. Encouragement of Arts, Manufacturers and Commerce 12.

BECH-ANDERSEN, J. (1985). Alkaline building materials and controlled moisture conditions as causes for the occurrence of the dry rot fungus in buildings. Mat. und Org. 20, 301 - 309.

BECH-ANDERSEN, J. (1987). Production, function and neutralisation of oxalic acid produced by the dry rot fungus and other brown rot fungi. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/1330.

BECH-ANDERSEN, J. (1991). The dry rot fungus and other fungi in houses. Holbaek, Denmark ; S.M. Olsen.

BECKER, G.H.R. (1968). Protection of timber - an introduction to some problems. In : Biodeterioration of materials. Microbiological and allied aspects (ed. A.H. Walters &

J.J. Elphick), pps 205 - 222. Amsterdam, Holland ; Elsevier Publishing Company.

BENHAMOU, N., OUELLETTE, G.B., LA FONTAINE, J.G. & JOLY, J.R. (1985). Use of monoclonal antibodies to detect a phytotoxic glycopeptide produced by Ophiostoma ulmi, the Dutch Elm disease pathogen. Can. J. Bot. 63, 1177 - 1184.

BENT, K.J. (1967). Electrophoresis of proteins of 3 Penicillium species on acrylamide gels. J. Gen. Microbiol. 49, 195 - 200.

BJURMAN, J. & KRISTENSSON, J. (1992). Analysis of volatile emissions as an aid in the diagnosis of dry rot. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/2393-92.

BLUM, H., BEIER, H. & GROSS, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93 - 99.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72, 248 - 254.

BRANLEY, R.R. (1803). Trans. Soc. Encour. Arts, Manufacturers and Commerce. 21, 298.

BRASIER, C.M. (1984). Intermycelial recognition systems in Ceratocystis ulmi : their physiological properties and ecological importance. In : The ecology and physiology of the fungal mycelium (British Mycological Symposium) (ed. D.H. Jennings & A.D.M. Rayner), pps 451 - 507. Cambridge, U.K. ; Cambridge University Press.

BRAVERY, A.F. & GRANT, C. (1985). Studies on the growth of Serpula lacrymans (Schumacher ex Fr.) Gray. Mat. und Org. 20, 171 - 191.

BRAVERY, A.F., BERRY, R.W., CAREY, J.K. & COOPER, D.E. (1987). Recognising wood rot and insect damage in buildings. Princes Risborough, U.K. ; Building Research Establishment, Department of the Environment.

BRITTON, T.A. (1875). A treatise on the Origin, Progress, Prevention and cure of dry rot in timber. London.

BROWN, J., FAHIM, M.M. & HUTCHINSON, S.A. (1968). Some effects of atmospheric humidity on the growth of Serpula lacrimans. Trans. Br. Mycol. Soc. 51, 507 - 510.

BROWNLEE, C. & JENNINGS, D.H. (1981). Further observations on tear or drop formation by mycelium of Serpula lacrimans. Trans. Br. Mycol. Soc. 77, 33 - 40.

BROWNLEE, C. & JENNINGS, D.H. (1982). Pathway of translocation in Serpula lacrimans. Trans. Br. Mycol. Soc. 79, 401 - 407.

BRUCE, A. (1983). Biological control of internal decay in creosoted distribution poles. Ph.D. thesis. Dundee College of Technology.

BUHLER, G.C.W. (1845). Der laufende Schwamm in den Gebauden, seine Entstehung, seine Vertilgung und die sichersten Mittel, sein Hervorbrechen zu verhindern. Stuttgart, Germany ; Schweizerbart'sche Verlagsbuchhandlung.

BURNETT, J.H. (1979). Fundamentals of Mycology. London, U.K. ; Edward Arnold.

BURNETTE, W.N. (1981). "Western blotting" : electrophoretic transfer of proteins from sodium dodecyl sulphate - polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated Protein A. Anal. Biochem. 112, 195 - 203.

BURNIE, J.P., MATTHEWS, R.C., CLARK, I. & MILNE, L.J.R. (1989). Immunoblot fingerprinting Aspergillus fumigatus. J. Immunol. Methods, 118, 179 - 186.

BUTLER, G.M. (1957). The development and behaviour of mycelial strands in Merulius lacrymans (Wulf.) Fr.
1. Strand development during growth from a food base through a non-nutrient medium. Annals of Botany 21, 523 - 537.

BUTLER, G.M. (1958). The development and behaviour of mycelial strands in Merulius lacrymans (Wulf.) Fr. 11. Hyphal behaviour during strand formation. Annals of Botany 22, 219 - 236.

BUTTERFIELD, B.G. & MEYLAN, B.A. (1980). Three dimensional structure of wood. An ultrastructural approach. London, U.K. ; Chapman and Hall.

CARTER, J.P. & LYNCH, J.M. (1991). Substrate dependent variation in the protein profile and antigens of Trichoderma harzianium. Enzyme Microbiol. Tech. 13, 537 - 543.

CARTWRIGHT, K.St.G. (1929). A satisfactory method of staining fungal mycelium in wood. Annals of Botany 43, 412 - 413.

CARTWRIGHT, K.St.G. & FINDLAY, W.P.K. (1958). Merulius lacrymans (Wulf.) Fr - the Dry rot fungus. In : Decay of timber and its prevention, pps 204 - 209. London, U.K. ; Her Majesty's Stationary Office.

CATTY, D. (1988). Properties of antibodies and antigens. In : Antibodies. Vol. 1 A practical approach (ed. D. Catty), pps 1 - 18. Oxford, U.K. ; IRL Press.

CATTY, D. & RAYKUNDALIA, C. (1989). ELISA and related techniques. In : Antibodies. Vol. 1 A practical approach (ed. D. Catty), pps 137 - 167. Oxford, U.K. ; IRL Press.

CHEN, W., HOY, J.W. & SCHNEIDER, R.W. (1991). Comparisons of soluble proteins and isozymes for seven Pythium species and applications of the biochemical data to Pythium systematics. Mycol. Res. 95, 548 - 555.

CHET, I., RETIG, N. & HENIS, Y. (1972). Changes in total soluble proteins and in some enzymes during nonphogenesis of Sclerotium rolfsii. J. Gen. Microbiol. 72, 451 - 456.

CLAPHAM, A.R. & NICHOLSON, B.E. (1975). Oxford Book of Trees. Oxford, U.K. ; Oxford University Press.

CLARE, B.G. (1963). Starch gel electrophoresis of proteins as an aid in identifying fungi. Nature 200, 803 - 804.

COBB, C.E. (1982). The non-enzymatic decomposition of cellulose by the brown-rot fungus Gleophyllum trabeum. Master's Thesis, Michigan Tech. Univ., 1 - 125.

COGGINS, C.R. (1976). Growth patterns of Serpula lacrimans, the dry rot fungus. B.W.P.A. annual convention, 1 - 10.

COGGINS, C.R. (1977). Aspects of the growth of Serpula lacrimans, the dry rot fungus. Ph.D thesis, Univ. of Liverpool.

COGGINS, C.R. (1980). Serpula lacrymans - the true dry rot fungus. In : Decay of timber in buildings, pps 34 - 35. East Grinstead, U.K. ; Rentokil Ltd.

COGGINS, C.R. & JENNINGS, D.H. (1975). A cabinet designed for fungal growth studies and an example of its use in investigating the sensitivity of Serpula lacrimans to zinc oxychloride. Int. Biodeterior. Bull. 11, 64 - 66.

COWLING, E.B. (1961). Comparative biochemistry of the decay of sweetgum by white-rot fungi. US Dept. of Agric. Techn. Bulletin 1258, 1 - 79.

COWLING, E.B. & MERRILL, W. (1966). Nitrogen in wood and its role in wood deterioration. Can. J. Bot. 44, 1539 - 1554.

CYMOREK, S. & HEGARTY, B. (1986). Differences among growth and decay capacities of 25 old and new strains of the dry rot fungus Serpula lacrymans using a special test arrangement. Mat. und Org. 21, 237 - 249.

DEACON, J.W. (1984). Introduction to modern mycology. Oxford, U.K. ; Blackwell Scientific Publications.

DESCH, H.E. & DINWOODIE, J.M. (1981). Timber; its structure, properties and utilisation. 6th Edition. London, U.K. ; The MacMillan Press Ltd.

DEWEY. F.M., M^{ac}DONALD, M.M. & PHILLIPS, S.I. (1989). Development of monoclonal-antibody -ELISA, -DOT-BLOT and -DIP-STICK immunoassays for Humicola lanuginosa in rice. J. Gen. Microbiology 135, 361 - 374.

DILLER, J.D. & KOCH, E.J. (1959). Weight losses of 40 wood species exposed to Poria incrassata and Merulius lacrymans. Forest Products Journal, 298 - 302.

DOI, S. (1989). Evaluation of preservative-treated wooden sills using a fungus cellar with Serpula lacrymans (Fr.) Gray. Mat. und Org. 24, 217 - 225.

DOI, S., SATO, M. & ARIMA, T. (1982). The measurement of temperature and relative humidity of a house damaged by Serpula lacrymans. Proc. of the Hokkaido Branch of the Japan Wood Research Society 14, 69 - 73.

DOI, S. & TOGASHI, I. (1990). Utilisation of soil components by Serpula lacrymans. Mat. und Org. 25, 201 - 209.

EAMUS, D. & JENNINGS, D.H. (1986). Turgor and fungal growth: studies on water relations of mycelia of Serpula lacrimans and Phallus impudicus. Trans. Br. Mycol. Soc. 86, 527 - 535.

ELLIOTT, C.G., ABOU-HEILAH, A.N., LEAKE, D.L. & HUTCHINSON, S.A. (1979). Analysis of wood decaying abilities of monokaryons and dikaryons of Serpula lacrymans. Trans. Br. Mycol. Soc. 73, 127 - 133.

ELLIOTT, M.L. & WATKINSON, S.C. (1989). The effect of α-amino-isobutyric acid on wood decay and wood spoilage fungi. International Biodeterioration 25, 355 - 371.

ERIKSSON, K.-E. L. (1985). Swedish developments in biotechnology based on lignocellulosic materials. Adv. in Biochem. Eng. 20, 193 - 204.

ERIKSSON, K.-E. L., BLANCHETTE, R.A. & ANDER, P. (1991). Microbial and enzymatic degradation of wood and wood components. Berlin, Germany ; Springer-Verlag.

ERIKSSON, K.-E. & PETTERSSON, B. (1975). Extracellular enzyme system utilised by the fungus Sporotrichum pulverulentum (Chrysosporium lignonum) for the breakdown of cellulose. Eur. J. Biochem. 51, 213 - 218.

ERIKSSON, K.-E. & WOOD, T.M. (1985). Biodegradation of cellulose. In : Biosynthesis and biodegradation of wood components (ed. T. Higuchi), pps 469 - 503. London, U.K. ; Academic Press Inc. Ltd.

ESSER, P.M. & TAS, A.C. (1992). Detection of dry rot by air analysis. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/2399-92.

FALCK, R. (1906). Uber den Hausschwamm. Z. Hyg. Infektionskrankh. 25, 478 - 505.

FALCK, R. (1912). Die Merulius Faule der Bauholzes. In : Hausschwammforschungen (ed. A. Moller), Vol. 6, pps 1 - 405. Germany, Jena ; Verlag v. Gustav Fischer.

FALCK, R. (1913). Die Fruchtkorperbildung der im Hause vorkommenden holzzerstorenden Pilze in Reinkulturen und ihre Bedingungen. Mykologische Untersuchungen und Berichte 1, 47 - 66.

FINDLAY, W.P.K. (1974). Wood rotting fungi. Trans. Bot. Soc. Edinb. 42, 181 - 186.

FOSTER, J.W. (1949). Chemical activities of fungi. New York, U.S.A. ; Academic Press.

FRANKLAND, J.C., BAILEY, A.D., GRAY, T.R.G. & HOLLAND, A.A. (1981). Development of an immunological technique for estimating mycelial biomass of Mycena galopus in leaf litter. Soil Biol. Biochem. 13, 87 - 92.

FRIES, E.M. (1821). Systema Mycologicum, Sistens Fungorum Ordines, Genera et Species, Vol.1, pp. 1 - 520. Gryphiswaldiae.

FUKUDA, K. & HARAGUCHI, T. (1985). Successive changes of lignin through all the stages of wood decay by the brown rot fungus Tyromyces palustris. Tokyo Noko Daigaku Nogakubu Enshurin Hokoku 21, 115 - 121.

GALBRAITH, D.N. (1992). Molecular analysis and detection of Heterobasidion annosum. Ph.D thesis, Dundee Institute of Technology.

GANDER, J.E. (1974). Fungal cell wall glycoproteins and peptido-polysaccharides. Ann. Rev. Microbiol. 28, 103 - 119.

GARCIA-MENDOZA, C., AVELLAN, M.A. & NOVAES-LEDIEV, M. (1987). Differentiation and wall chemistry of Agaricus bisporus vegetative and aggregated mycelia. Arch. Microbiol. 148, 68 - 71.

GEOGRAPHICAL DIGEST 1990-1991 (1990). Heineman-Philip Atlases. Oxford, U.K.: George Philip Ltd.

GLANCY, H., BRUCE, A., BUTTON, D., PALFREYMAN, J.W. & KING, B. (1989). Application of immunological methods to the analysis and detection of Lentinus lepideus Fr. Int. Res. Gp. on Wood. Pres., Doc. no. : IRG/WP/1422.

GLANCY, H. (1990). Detection and analysis of the wood decay fungus Lentinus lepideus Fr. using immunological probes. Ph.D thesis, Dundee Institute of Technology.

GLASS, W.F., BRIGGS, R.C. & HNILICA, L.S. (1981). Use of lectins for detection of electrophoretically separated glycoproteins transferred onto nitrocellulose. Anal. Biochem. 115, 219 - 224.

GOODELL, B.S. & JELLISON, J. (1986). Detection of a brown-rot fungus using serological assays. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/1305.

GOODELL, B.S., DANIEL, G., JELLISON, J. & NILSSON, T. (1988). Immunolabelling of Poria placenta in wood. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/1361.

GRAHAM, R.C. & KARNOVSKY, M.J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney ultrastructural cytochemistry by a new technology. J. Histochem. Cytochem. 14, 291 - 302.

GRAVES, D.C., McNABB, S.J.N., WORLEY, M.A., DOWNS, T.D. & IVEY, M.H. (1986). Analyses of rat Pneumocystis carinii antigens recognised by human and rat antibodies by using western blotting. Infectious Immunology 54, 96 - 103.

GRAY, S.F. (1821). A natural arrangement of British Plants according to their relations to each other. Vol.1, p.637. London, U.K.: Baldin, Craddock and Joy.

GRIFFIN, D.H. (1981). Fungal physiology. New York, U.S.A. ; John Wiley & Sons.

HAINEY, S.D. (1992). An investigation of the durability of UK grown softwood distibution poles CCA-treated by sap-displacement. PhD thesis. Dundee Institute of Technology.

HALL, R., ZENTMEYER, G.A. & ERWIN, D.C. (1969). Approach to taxonomy of Phythophthora through acrylamide gel-electrophoresis of proteins. Phytopathology 59, 770 - 774.

HANCOCK, K. & TSANG, V.C.W. (1983). India ink staining of protein on nitro-cellulose paper. Anal. Biochem. 133, 157 - 162.

HANSEN, E.M., BRASIER,C.M., SHAW, D.S. & HAMM, P.B. (1986). The taxonomic structure of Phytophthora megasperma: evidence

for emerging biological species groups. Trans. Br. Mycol. Soc. 87 (4), 557 - 573.

HARDHAM, A.R., SUZAKI, E. & PERKIN, J.L. (1986). Monoclonal antibodies to isolate-, species- and genus- specific components on the surface of zoospores and cysts of the fungus Phytophthora cinnamomi. Can. J. Bot. 64, 311 - 321.

HARMSEN, L. (1960). Taxonomic and cultural studies of brown spored species of the genus Merulius. Friesia 6 (4), 233 - 277.

HARTIG, R. (1878). Die Zersetzungerscheinnungen des Holzes der Nadelbaum und der Eiche. Germany, Berlin.

HARTIG, R. (1885). Die Zerstorungen des Bauholzes durch Pilze. I. Der echte Hausschwamm (Merulius lacrymans Fr.). Germany, Berlin: Verlag v. Julius Springer.

HAYASHI, O., YAMADA, H., YADOMAE, T. & MIYAZAKI, T. (1978). Purification and partial characterisation of the serologically active substance from Absidia cylindrospora. J. Gen. Microbiol. 106, 289 - 295.

HEARN, V.M. & M^{ac}KENZIE, D.W.R. (1979). The preparation and chemical composition of fractions from Aspergillus fumigatus wall and protoplasts possessing antigenic activity. J. Gen. Microbiol. 112, 35 - 44.

HEARN, V.M. & M^{ac}KENZIE, D.W.R. (1980). The preparation and partial purification of fractions from mycelial fungi with antigenic activity. Molecular immunology 17, 1097 - 1103.

HEARN, V.M., GRIFFITHS, B.L. & GORIN, P.A.J (1989). Structural analysis of water-soluble fractions obtained from Aspergillus fumigatus mycelium. Glycoconjugate 6, 85 - 100.

HEARN, V.M., WILSON, E.V., LATGE, J.-P., & M^{ac}KENZIE, D.W.R. (1990). Immunochemical studies of Aspergillus fumigatus mycelial antigens by polyacrylamide gel electrophoresis and Western blotting techniques. J. Gen. Microbiol. 136, 1525 - 1535.

HEGARTY, B. (1991). Factors affecting the fruiting of the Dry Rot fungus Serpula lacrymans. In : Serpula lacrymans. Fundamental biology and control strategies (eds D.H. Jennings & A.F. Bravery), pps 39 - 53. Chichester, U.K. ; John Wiley & Sons.

HEGARTY, B. & SCHMIDT, U. (1988). Basidiospore structure and germination of Serpula lacrymans and Coniophora puteana. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/1340.

HIGHLEY, T.L. (1975). Properties of cellulases of two brown-rot and two white-rot fungi. Wood and Fiber 6, 275 - 281.

HIGHLEY, T.L. (1978). Degradation of cellulose by culture filtrates of Poria placenta. Mat. und Org. 12, 161 - 174.

HILL, J.B., OVERHOLTS, L.O., POPP, H.W. & GROVE, A.R. (1960). Botany. New York, U.S.A.: McGraw-Hill Book Co. Inc.

HORNUNG, U. & JENNINGS, D.H. (1981). Light and electron microscopical observations of surface mycelium of Serpula lacrimans : stages of growth and hyphal nomenclature. Nova Hedwigia 34, 101 - 126.

HSU, U.-M. & SOBAN, E. (1982). Colour modification of dianinobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. J. Histochem. Cytochem. 30, 1079 - 1082.

HUDSON, H.J. (1986). Lignin degradation. In : Fungal Biology, pps 88 -96. Baltimore, U.S.A ; Edward Arnold.

HUECK, H.J. (1965). The Biodeterioration of materials as part of hylobiology. Mat. und Org. 1, 5 - 34.

HUNSLEY, D. & BURNETT, J.H. (1970). The ultrastructural architecture of the walls of some hyphal fungi. J. Gen. Microbiol. 62, 203 - 218.

HUNT, R.S. & COBB, F.W. (1971). Selective medium for the isolation of wood-rotting Basidiomycetes. Can. J. Bot. 49, 2064 - 2065.

JACKMAN, P.J.H., FELTHAM, R.K.A. & SNEATH, P.H.A. (1983). A program in BASIC for numerical taxonomy of micro-organisms based on electrophoretic protein patterns. Microbios Letters 23, 87 - 98.

JANE, F.W. (1956). The Structure of Wood. London, U.K. ; Adam and Charles Black.

JELLIS, G.J., SMITH, D.B. & SCOTT, E.S. (1990). Identification of Sclerotinia spp. on Vicia faba. Mycol. Res. 94, 407 - 409.

JENG, R.S. & HUBBES, M. (1983). Identification of aggressive and non-aggressive strains of Ceratocystis ulmi by polyacrylamide gradient gel electrophoresis of intramycelial proteins. Mycotaxon 17, 445 - 455.

JENNINGS, D.H. (1984). Water flow through mycelia. In : The ecology and physiology of the fungal mycelium (eds D.H. Jennings & A.D.M. Rayner), pps 143 - 161. Cambridge, U.K. ; Cambridge University Press.

JENNINGS, D.H. & BRAVERY, A.F. (1991). Serpula lacrymans. Fundamental biology and control strategies. Chichester, U.K. ; John Wiley and Sons Ltd.

JENNINGS, L. & WATKINSON, S.C. (1982). Structure and development of mycelial strands in Serpula lacrimans. Trans. Br. Mycol. Soc. 78, 465 - 474.

JOHNSON, D.A., GAUTSCH, J.W., SPORTSMAN, J.R. & ELDER, J.H. (1984). Improved technique utilising nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Techn. 1, 3 - 8.

JOHNSTONE, A. & THORPE, R. (1982). Immunocytochemistry in Practice. Oxford, U.K. ; Blackwell Scientific Publishers.

KERSTERS, K. (1985). Numerical methods in the classification of bacteria by protein electrophoresis. In : Computer assisted bacterial systematics (eds M. Goodfellow, D. Jones & F.G. Priest), pps 337 - 368. London, U.K. ; Academic Press.

KIJIMOTO-OCHIAI, S., KATAGIRI, Y.U. & OCHIAI, H. (1985). Analysis of N-linked oligosaccharide chains of glycoproteins on nitrocellulose sheets using lectin peroxidase reagents. Anal. Biochem. 147, 222 - 229.

KING, B. (1981). The durability of timber and timber products. Bull. Inst. Corrosion Science and Technology, 5 - 11.

KIRK, T.K. (1971). Effects of microorganisms on lignin. Ann. Rev. Phytopath. 9, 185 - 210.

KIRK, T.K. (1973). The chemistry and biochemistry of decay. In : Wood deterioration and its preservation by preservative treatments (Degradation and protection of wood) (ed. D.D. Nicholas), vol. 1, pps 149 - 181. Syracuse, U.S.A. ; Syracuse University, Syracuse Wood Science Service.

KIRK, T.K. (1975). Effects of a brown rot fungus, Lenzites trabea, on lignin in spruce wood. Holzforschung 29, 99 - 107.

KIRK, T.K. (1984). Degradation of lignin. In : Microbial decomposition of wood (ed. D.T. Gibson), pps 399 - 437. Basel, Switzerland ; Marcel Dekker Inc.

KOCH, A.-P. (1991). The current status of dry rot in Denmark. In : Serpula lacrymans : Fundamental biology & control strategies (eds D.H. Jennings & A.F. Bravery), pps 147 - 154. Chichester, U.K. ; John Wiley & Sons.

KOENIGS, J.W. (1974a). Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown-rot basidiomycetes. Wood and Fiber 6, 66 - 80.

KOENIGS, J.W. (1974b). Production of hydrogen peroxide by wood rotting fungi in wood and its correlation with weight loss, depolymerisation and pH changes. Arch. Microbiol. 99, 129 - 145.

KOLLMAN, F.F.P. & COTE, W.A. (1968). Principles of wood science and technology. 1. Solid Wood. London, U.K. ; George Allen and Unwin Ltd.

KYHSE-ANDERSEN, J. (1984). Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Meth. 10, 203 - 209.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680 - 685.

LAIDLAW, R.A. & SMITH, G.A. (1965). The proteins of the timber of Scots Pine (Pinus sylvestris). Holzforschung 19, 129 - 134.

LANGVAD, F. & GOKSOYR, J. (1965). Effect of supraoptimal temperatures on Merulius lacrymans. Physiol. Planta 20, 702 - 712.

LEARY, J.J., BRIGATI, D.J. & WARD, D.C. (1983). Rapid and sensitive colorimetric method for visualising biotin-labelled DNA probes hybridised to DNA or RNA immobilised on nitrocellulose : Bio-blots. Proc. Natl. Acad. Sci. USA 80, 4045 - 4049.

LEVI, M.P. & COWLING, E.B. (1969). Role of nitrogen in wood deterioration. VII. Physiological adaptation of wood destroying and other fungi to substrates deficient in nitrogen. Phytopathology 59, 460 - 468.

MAAS, E.M., VAN ZYL, E., STEYN, P.L. & KOTZE, J.M. (1990). Comparison of soluble proteins of Gaeumannomyces graminis var. tritici and Phialophora spp. by polyacrylamide gel electrophoresis. Mycol. Res. 94, 78 - 82.

MacDONALD, M.J., LIWICKI, R.D.J. & BRODA, P. (1984). Physiological changes in Phanerochaete chrysosporium during the onset of ligninolytic activity. Appl. Biochem. Biotechn. 9, 381 - 382.

MacDONALD, M.M., DUNSTAN, R.H. & DEWEY, F.M. (1989). Detection of low-M_r glycoproteins in surface washes of some fungal cultures by gel-filtration HPLC and by monoclonal antibodies. J. Gen. Microbiol. 135, 375 - 383.

MADSEN, B. & ADELHOEJ, J. (1989). Testing of wooden constructions in buildings. 7th Int. Symposium on Nondestructive testing of wood.

MALTERUD, K.E., BREMNES, T.E., FAEGRI, A., MOE, T., DUGSTAD, E.K.S., ANTHONSEN, T. & HENRIKSEN, L.M. (1985). Flavenoids from the wood of Salix caprea as inhibitors of wood-destroying fungi. J. Nat. Products 48, 559 - 563.

MARCHANT, R. & SMITH, D.G. (1968). A serological investigation of hyphal growth in Fusarium culmorum. Archiv. fur Mikrobiologie 63, 85 - 94.

MARGOLIS, J. & KENRICK, K.G. (1967). Polyacrylamide Gel-electrophoresis across a molecular sieve gradient. Nature 214, 1334 - 1336.

MARGOLIS, J. & KENRICK, K.G. (1968). Polyacrylamide Gel Electrophoresis in a continuous molecular sieve gradient. Anal. Biochem. 25, 347 - 362.

MARSDEN, H.S., STOW, N.D., PRESTON, V.G., TIMBURY, M.C. & WILKIE, N.M. (1978). Physical mapping of herpes simplex virus-induced polypeptides. J. Virol. 28, 624 - 642.

MARSDEN, W.E. & MARSDEN, V.M. (1989). The Forest based industries of Sweden. In : Going into Europe, pps 49 - 51. Edinburgh, U.K. ; Oliver & Boyd.

M^{c}CUTCHEON, S. (1989). Analysis of hyphal interaction antigens. B.Sc. 4 Hons. project, Dundee Institute of Technology.

M^{c}DOWELL, H. (1992). Molecular analysis of wet rot organisms; application to conservation of maritime artefacts. Ph.D. thesis, Dundee Institute of Technology.

M^{c}DOWELL, H. & PALFREYMAN, J.W. (1992). Molecular analysis of the Basidiomycete Coniophora puteana. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/1534-92.

M^{c}GADEY, J. (1970). A tetrazolium method for non-specific alkaline phosphatase. Histochemie 23, 184 - 184.

MONTGOMERY, R.A.P. (1982). The role of polysaccharidase enzymes in the decay of wood by basidiomycetes. In : Decomposer Basidiomycetes, their biology and ecology (eds J.C. Frankland, J.N. Hedger & M.J. Swift), pps 51 - 65. Cambridge, U.K. ; Cambridge University Press.

MYERS, N. (1985). The Gaia Atlas of Planet Management for Tomorrow's World (ed. N. Myers). London, U.K. ; Pan Books.

NILSSON, T. (1985). Defining fungal types - final proposal. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/1355

NOBLES, M.K. (1965). Identification of cultures of wood inhabiting Hymenomycetes. Can. J. Bot. 43, 1097 - 1139.

NUSS, I., JENNINGS, D.H. & VELTKAMP, C.J. (1991). Morphology of Serpula lacrymans. In : Serpula lacrymans. Fundamental

biology and control strategies (eds D.H. Jennings & A.F. Bravery), pps 9 - 38. Chichester, U.K. ; John Wiley & Sons.

PALFREYMAN, J.W., BRUCE, A., BUTTON, D., GLANCY, H., VIGROW, A. & KING, B. (1987). Immunological methods for the detection and characterisation of wood decay basidiomycetes. In : Biodeterioration (eds D.R. Houghton, R.N. Smith & H.O.W. Eggins), vol. 7, pps 709 - 713. London, U.K. ; Elsevier Publishing Company.

PALFREYMAN, J.W., GLANCY, H., BUTTON, D., VIGROW, A., SCORE, A. & KING, B. (1988). Use of immunoblotting for the analysis of wood decay Basidiomycetes. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/2307.

PALFREYMAN, J.W., TIMMONS, H., VIGROW, A., BUTTON, D. & KING, B. (1990). Identification of wood decay Basidiomycetes using SDS-PAGE. Int. Res. Gp. on Wood Pres.

PALO, T.P. (1984). Distribution of birch (Betula spp.), willow (Salix spp.) and poplar (Populus spp.) secondary metabolites and their potential role as chemical defence against herbivores. J. Chem. Ecol. 10, 499 - 520.

PEGLER, D.N. (1991). Taxonomy, identification and recognition of Serpula lacrymans. In : Serpula lacrymans. Fundamental biology and control strategies (eds D.H. Jennings & A.F. Bravery), pps 1 - 7. Chichester, U.K. ; John Wiley & Sons Ltd.

PEPYS, J. & LONGBOTTOM, J.L. (1979). Immunological methods in mycology. In : Application of immunological methods. Handbook of experimental immunology (ed. D.M. Weir), vol. 3, pps 41.1 - 41.27. Oxford, U.K. ; Blackwell Scientific Publications.

PUGH, D. & CAWSON, R.A. (1977). The cytochemical localisation of acid hydrolases in four common fungi. Cellular and Molecular Biology 22, 125 - 132.

RAMSBOTTOM, J. (1937). Dry rot in ships. Essex Naturalist 25, 231 - 267.

RAYNER, A.D.M. & BODDY, L. (1988). Fungal decomposition of wood : its biology and ecology. Chichester, U.K. ; John Wiley and Sons.

READMAN, M.J. & MAYERS, F.M. (1986). The Dynamic Landscape. Edinburgh, U.K. ; Oliver and Boyd.

ROITT, I., BROSTOFF, J. & MALE, D. (1985). Immunology. London, U.K. ; Gower Medical Publishing.

ROSENBERGER, R.F. (1976). The Cell Wall. In : The filamentous fungi. Vol. II Biosynthesis and metabolism (eds J.E. Smith & D.R. Berry), pps 328 - 344. London, U.K. ; Edward Arnold.

SAVORY, J.G. (1954). Breakdown of timber by Ascomycetes and Fungi Imperfecti. Ann. Appl. Biol. 41, 336 - 347.

SAVORY, J.G. (1980). Treatment of outbreaks of dry rot, Serpula lacrymans. B.W.P.A. News Sheet 160.

SCHMIDT, O. & KEBERNIK, U. (1989). Characterisation and identification of the dry rot fungus Serpula lacrymans by polyacrylamide gel electrophoresis. Holzforschung 43, 195 - 198.

SCHUMACHER, M.J., M^{c}CLATCHY, J.K., FARR, R.S. & MINDEN, P. (1975). Primary interaction between antibody and components of Allemaria. I. Immunological and chemical characteristics of labelled antigens. J. Allergy Clinical Immunology 56, 39 - 53.

SCOTT, P.H., (1991). Identification and detection of Phytophthora spp. causing root rot disease of raspberries. M. Phil. thesis. Scottish Crop Research Institute & Dundee Institute of Technology.

SEEHAN, G. & HEGARTY, B.M. (1988). A bibliography of the dry rot fungus, Serpula lacrymans. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/1337.

SEELIGER, H.P.R. (1982). Significance of immunological methods for diagnosis of fungal disease. Chemotherapy 28, 54 - 63.

SHAPIRO, A.L., VINUELA, E. & MAIZEL, J.V. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in

SDS and polyacrylamide gels. Biochemical Biophysical Research Communications 28, 815 - 21.

SLATER, G.G. (1965). Electrophoresis on a density gradient of polyacrylamide gel - apparatus and examples. Federation proceedings 24, 225.

SLATER, G.G. (1969). Stable pattern formation and determination of molecular size by pore-limit electrophoresis. Anal. Chem. 41, 1039 - 1041.

SMITH, H.S. (1948). Biological control of insect pests. In : The citrus industry II (eds L.D. Batchelor & H.D. Webber), pps 597 - 625. Los Angeles, U.S.A. ; University of California Press.

SMITH, R.S. (1965). Sterilisation of wood test blocks by volatile chemicals: effects on Lentinus lepideus. Trans. Br. Mycol. Soc. 48 (3), 341 - 347.

SNEATH, P.H.A. & SOKAL, R.R. (1985). The principles of numerical taxonomy : twenty-five years later. In : Computer assisted bacterial systematics (eds M. Goodfellow, D. Jones & F.G. Priest), pps 1 - 20. London, U.K. ; Academic Press Ltd.

SOUKUP, F. (1979). An interesting record of Serpula lacrimans in the Botanical Garden in Pruhonice near Prague. Ceska Mykol. 33, 94 - 98.

SPREY, B. (1988). Cellular and exocellular localisation of endocellulase in Trichoderma reesei. FEMS Microbiology Letters 55, 283 - 294.

STALPERS, J.A. (1978). Identification of wood inhabiting fungi in pure culture. Studies in Mycology 16, 1 - 248.

STASZCZAK, M. & NOWAK, G. (1984). Proteinase pattern in Trametes versicolor in response to carbon and nitrogen starvation. Acta Biochim. Polonica 31, 431 - 437.

STOTT, D.I. (1989). Immunoblotting and dot blotting. J. Imm. Meth. 119, 153 - 187.

TAKAHASHI, M. & NISHIMOTO, K. (1985). Improved techniques designed for evaluation of fungicides in soil for control of dry

rot fungus Serpula lacrymans. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/2238.

TAYLOR, I.E.P. & CAMERON, D.S. (1973). Preparation and quantitative analysis of fungal cell walls : strategy and tactics. Ann. Rev. Microbiol. 27, 243 - 259.

THORNTON, J.D. (1985). The survival and wood rotting abilities of eight Serpula lacrymans strains incubated during the summer season within the subfloor spaces of two Australian buildings. Mat. und Org. 20, 109 - 119.

THORNTON, J.D. (1989). A new laboratory technique devised with the intention of determining whether, related to practical conditions, there should be a relationship between growth rate and decay capacity (of different strains) of Serpula lacrymans. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/1384.

THORNTON, J.D. & COLLETT, O. (1983). The growth and behaviour of Australian isolates of dry rot fungi, mainly Serpula lacrymans (Schum. ex. Fr.) S.F. Gray. A comparison with the world literature. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/1190.

THORNTON, J.D. & WAZNY, J. (1986). Comparitive susceptibility of sapwood blocks of Pinus sylvestris and Pinus radiata to decay by Serpula lacrymans. Holzforschung 40, 129 - 130.

TIMES CONCISE ATLAS OF WORLD HISTORY (1986). (ed. G. Barraclough). London, U.K. ; Times Books Ltd.

TOWBIN, H. & GORDON, J. (1984). Immunoblotting and dot immunobinding - current status and outlook. J. Imm. Meth. 72, 313 - 340.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350 - 4354.

TURIAN, G. (1983). Concepts of fungal differentiation. In : Fungal differentiation : a contemporary synthesis (ed. J.E. Smith), pps 1 - 18. New York, U.S.A. ; Marcel Dekker Inc.

VENABLES, C.E. & WATKINSON, S.C. (1989). Production and localisation of proteinases in colonies of timber-decaying basidiomycete fungi. J. Gen. Microbiol. 135, 1369 - 1374.

WALCZ, I., PACSA, A.S., EMODY, L. & SZABO, L.G. (1985). Detection of Sclerotinia sclerotiorum in sunflower by enzyme-linked immuno-sorbent assay (ELISA). Trans. Br. Mycol. Soc. 85, 485 - 488.

WATKINSON, S.C. (1971a). The mechanism of mycelial strand induction in Serpula lacrimans : a possible effect of nutrient distribution. New Phytol. 70, 1079 - 1088.

WATKINSON, S.C. (1971b). Phosphorus translocation in the stranded and unstranded mycelium of Serpula lacrymans. Trans. Br. Mycol. Soc. 57, 535 - 539.

WATKINSON, S.C. (1975). The relation between nitrogen nutrition and formation of mycelial strands in Serpula lacrimans. Trans. Br. Mycol. Soc. 64, 195 - 200.

WATKINSON, S.C. (1979). Growth of rhizomorphs, mycelial strands, coremia and sclerotia. In : Fungal walls and hyphal growth. British Mycological Society Symposia, vol 2 (eds J.H. Burnett & A.P.J. Trinci), pps 93 - 113. Cambridge, U.K. ; Cambridge University Press.

WATKINSON, S.C. (1984a). Morphogenesis of the Serpula lacrimans colony in relation to its function in nature. In : The ecology and physiology of the fungal mycelium. British Mycological Society Symposia, vol 8 (eds D.H. Jennings & A.D.M. Rayner), pps 165 - 184. Cambridge, U.K. ; Cambridge University Press.

WATKINSON, S.C. (1984b). Inhibition of growth and development of Serpula lacrimans by the non-metabolised amino acid analogue α-aminoisobutyric acid. FEMS Microbiol. Letters 24, 247 - 250.

WATKINSON, S.C., DAVISON, E.M. & BRAMAH, J. (1981). The effect of nitrogen availability on growth and cellulolysis by Serpula lacrimans. New Phytol. 89, 295 - 305.

WAZNY, J. & THORNTON, J.D. (1986). Comparitive laboratory testing of strains of the dry rot fungus Serpula lacrymans (Schum. ex. Fr.) S.F. Gray. II. The action of some wood preservatives in agar media. Holzforschung 40, 383 - 388.

WAZNY, & THORNTON, J.D. (1991). A comparison analysis of eight strains of Serpula lacrymans (Schum. ex. Fr.) S.F. Gray. Int. Res. Gp. on Wood Pres., Doc. no. : IRG/WP/2362.

WEBER, K. & OSBORN, M.J. (1969). The reliability of molecular weight determinations by dodecylsulphate-polyacrylamide gel electrophoresis. Biol. Chem. 224, 4406 - 4412.

WESTERMARK, U. & ERIKSSON, K.-E. (1975). Purification and properties of cellobiose: quinone oxidoreductase from Sporotrichum pulverulentum. Acta Chem. Scan. B29, 419 - 424.

WILKINSON, J.G. (1979). The deterioration and preservation of timber. In : Industrial Timber Preservation, pps 73 - 123. East Grinstead, U.K. ; Rentokil Library, Rentokil Ltd.

WONG, A.L. & WILLETTS, H.J. (1973). Electrophoretic studies of soluble proteins and enzymes of Sclerotinia species. Trans. Br. Mycol. Soc. 61, 167 - 178.

WOSTEN, H.A.B., MOUKHA, S.M., SIETSMA, J.H. & WESSELS, J.G.H. (1991). Localisation of growth and secretion of proteins in Aspergillus niger. J. Gen. Microbiol. 137, 2017 - 2023.

WULFEN, F.X. von (1781). In : Miscellanea Austriaca ad Botanicum, Chemiam et Historiam Naturalem Spectantia, vol 2 (ed N.J. Jacquin), pps 98 - 113. Vindobonae.

WYCOFF, K.L., JELLISON, J. & AYERS, A.R. (1987). Monoclonal antibodies to glycoprotein antigens of a fungal plant pathogen, Phytophthora megasperma f.sp. glycinea. Plant Physiol. 85, 508 - 515.

APPENDICES.

APPENDIX A

This appendix lists the reagents and chemicals mentioned in the project. All chemicals were obtained from Sigma Chemical Company Ltd unless stated otherwise.

A1 SUPPLIERS OF EQUIPMENT AND CHEMICALS.

BDH Ltd, Poole, Dorset, UK.
Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK, HP2 7TD.
Birchover Instruments Ltd, Bridge Road, Letchworth, Hertfordshire, UK, SG6 4ET.
Boehringer Mannheim Biochimica, Lewes, East Sussex, UK, EN7 1LG.
Fisons, FSA Laboratory Suppliers, Loughborough, UK.
Fluka Chemie A.G., CH-9470 Buchs, Switzerland.
Gallenkamp (Fisons).
Glen Creston Ltd, Stanmore, Middlesex, UK, HA7 1DA.
Millipore (UK) Ltd, Watford, Hertfordshire, UK, WD1 8YW.
Oxoid, UNIPATH, Basingstoke, UK.
Pharmacia-LKB Biotechnology, Milton Keynes, Buckinghamshire, UK, MK9 3HP.
Pierce, Rockford, Illinois, USA.
Scottish Antibody Production Unit, Common Health Service Agency for the Scottish Health Service, Law Hospital, Carluke, UK, ML8 5ES.
Sartorius Ltd, GB-Belmont, Surrey, UK, SM2 6JD.
Sigma Chemical Company Ltd, Poole, Dorset, UK, BH17 7TG.

A2 5% MALT EXTRACT/ 2% AGAR (both w/v).

5 g malt extract (Oxoid) and 2 g purified agar (Oxoid) were autoclaved (15 min, 15 psi) in 100 ml ultra-pure water (u-p), cooled to 50°C and either poured into sterile Petri dishes to a depth of 8 mm or used to prepare slopes in universal jars for stock cultures.

A3 5% (w/v) MALT EXTRACT BROTH.

5 g malt extract broth (Oxoid) in 100 ml u-p water was autoclaved (15 min, 15 psi), cooled to 50°C and poured into sterile Petri dishes to a depth of 6 mm.

A4 SABOURAUD'S LIQUID MEDIUM SUBSTITUTE.

0.9% sodium chloride (w/v)/2% glucose (w/v), pH 7.4 (Oxoid, personal communication).

0.9 g NaCl in 100 ml u-p water was adjusted to pH 7.4, autoclaved (15 psi, 15 min) in 100 ml u-p water and cooled to 50°C. 2 g glucose was aseptically added, dissolved and the medium either was poured into sterile Petri dishes (0% TN) to a depth of 6 mm or was used with 24.8% mycological peptone (A5) to give media with TN contents of 2.36%, 0.127% and 0.03%.

A5 24.8% (w/v) MYCOLOGICAL PEPTONE.

24.8 g mycological peptone (9.5% TN) (Oxoid) was autoclaved (15 psi, 15 min) with 100 ml u-p water, cooled to 50°C and either poured directly into sterile Petri dishes (9.5% TN) or diluted with Sabouraud's liquid medium substitute (A4) prior to plate preparation.

A6 DIFFERENTIAL STAINING OF FUNGUS IN WOOD.

10 µm TLS wood sections were cut into PBS (A7). individual sections were flooded with 1% aqueous safranin for 40 sec; washed with u-p water till the water was clear; flooded with picro-aniline blue stain; heated until the liquid just boiled and immediately flooded with a large volume of u-p water; and washed until excess colour had gone. Dehydration in 70% ethanol (2 x 30 sec) and absolute ethanol (30 sec) preceded a clearing stage in clove oil (30 sec) and a xylene wash (30 sec) prior to mounting in DPX.

A7 PHOSPHATE BUFFERED SALINE 10mM, pH 7.4 (PBS).

1. 0.02M solutions of sodium di-hydrogen phosphate (stock solution A) and di-sodium hydrogen ortho-phosphate (stock solution B) were prepared.
2. Phosphate buffer 10mM, pH 7.4 (PB) was obtained by mixing 19 ml of stock solution A and 81 ml of stock solution B with 100 ml u-p water.
3. PBS was obtained by addition of NaCl to PB to a final concentration of 0.15M.

A8 FREUND'S COMPLETE ADJUVANT.

Each ml contains 1 mg of Mycobacterium tuberculosis (H37Ra, ATCC 25177), heat killed and dried, 0.85 ml paraffin oil and 0.15 ml mannide mono-oleate.

A9 FREUND'S INCOMPLETE ADJUVANT.

Each ml contains 0.85 ml paraffin oil and 0.15 ml mannide mono-oleate.

A10 DIG GLYCAN DETECTION KIT, Boehringer Mannheim, Catalogue number 1142372.

A11 BUFFERS FOR IMMUNOLOGICAL TECHNIQUES.

Each component of the buffer has been given an abbreviation which will subsequently be used throughout the text.

Component		Source
Bovine serum albumin	BSA	Sigma
Newborn calf serum	NCS	Sigma
Normal donkey serum	NDS	SAPU
Normal goat serum	NGS	SAPU
Non-fat dried milk (Blotto)	B	Cadbury's Marvel
Tween 20	T	Sigma

The numbers used with the abbreviations in the following table of buffers refer to the percentage concentration (w/v or v/v) of the particular component in PBS.

Blocking agent	Blocking buffer	Dilution buffer	Wash buffer
B	PBS/5B	PBS/5B	PBS/5B
BSA	PBS/5BSA	PBS/5BSA	PBS/5BSA
	PBS/3BSA	PBS/3BSA	PBS/3BSA
		PBS/1BSA	PBS/1BSA
NCS	PBS/5NCS	PBS/5NCS	PBS/5NCS
	PBS/3NCS	PBS/3NCS	PBS/3NCS
		PBS/1NCS	PBS/1NCS
NDS	PBS/5NDS	PBS/5NDS	PBS/5NDS
	PBS/3NDS	PBS/3NDS	PBS/3NDS
		PBS/1NDS	PBS/1NDS
NGS	PBS/5NGS	PBS/5NGS	PBS/5NGS

	PBS/3NGS	PBS/3NGS	PBS/3NGS
		PBS/1NGS	PBS/1NGS
T	PBS/0.5T	PBS/0.05T	PBS/0.05T
T/B	PBS/0.5T/5B	PBS/0.05T/5B	PBS/0.05T/5B
T/NCS	PBS/0.5T/10NCS	PBS/0.05T/5NCS	PBS/0.05T/5NCS
			PBS/0.05T
			PBS
	PBS/0.5T/5NCS	PBS/O.O5T/5NCS	PBS/0.05T

A12 MEMBRANES USED IN IMMUNOLOGICAL TECHNIQUES.

Membranes were handled with gloves and forceps; and cut with a guillotine.

Nitrocellulose (Bio-Rad Transblot Transfer Medium) was washed twice in u-p water and dried (37°C) prior to use.

Immobilon (Millipore PVDF membrane) was dipped for 30 sec in analar methanol and transferred to Anode buffer 2 (A 28.1) prior to use.

A13 CHROMOGENIC SUBSTRATES.

All substrate solutions were prepared immediately prior to use. 10 ml of substrate were prepared per 15 cm^2 of membrane. The reaction was stopped with 2 washes of u-p water and the membranes were dried at R/T.

1. 3,3'-diaminobenzidine tetra-hydrochloride (DAB) substrate solution.
   6 mg DAB was dissolved in 10 ml Tris (Trizma base) 50mM, pH 7.6 and 10 µl H_2O_2 (100 vols) was added prior to addition to the membrane. An orange/brown reaction product formed within 1 - 5 min after constant rocking.

2. DAB enhanced with nickel chloride (DAB/N) substrate solution.
   6 mg DAB was dissolved in 10 ml Tris 50mM, pH 7.6 and 1 ml 0.3% (w/v) nickel chloride was added. 10 µl H_2O_2 (100 vols) was added prior to addition of the substrate solution to the membrane. A black reaction product formed within 1 - 5 min after constant rocking.

3. 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium chloride (BCPIP/NBT) substrate solution.
50 mg BCPIP (A10) was dissolved in 1 ml dimethylformamide (BCPIP solution) and 100 mg NBT (A10) was dissolved in 1.3 ml 70% (v/v) dimethylformamide (NBT solution). Immediately prior to use of the substrate solution 37.5 µl BCPIP solution and 50 µl NBT solution were added to 10 ml Tris buffer 0.1M, pH 9.5 and the filter was immersed with no shaking. The dark purple reaction product formed within 60 min.

Tris buffer 0.1M, pH 9.5
Trizma hydrochloride (Tris-HCl) 0.1M was adjusted to pH 9.5; and $MgCl_2$ (0.05M) and NaCl (0.1M) were added.

A14 DOT IMMUNOBINDING PROTOCOL.

Wash	wash buffer	x 1
Block	blocking buffer	60 min, R/T, shake
Wash	wash buffer	x 2
Incubation with Ab1	Ab1 at required concentration in dilution buffer	60 min, R/T, shake
Wash	wash buffer	x 5
Incubation in HRP-Ab2	HRP-Ab2 at 1:200 in dilution buffer	60 min, R/T, shake
Wash	wash buffer	x 5
Wash	PBS	x 2
Detection of Ab:Ag complex	DAB substrate	
Wash	u-p water	x 2
Dry	30°C	

A15 BINDING OF FUNGAL LECTINS.

The following sugars were prepared in the appropriate buffers at a final concentration of 0.1M:

D(+)glucose.
D(+)mannose.
D(+)galactose.
N-acetyl-D-glucosamine.
N-acetyl-D-galactosamine.

A16 QUENCHING OF ENDOGENOUS PEROXIDASE IN THE ANTIGEN.

20 µl antigen supernatant at 25 mg/ml was added to 20 µl 1% (v/v) H_2O_2 (100 vols) in analar methanol and incubated at R/T for 10 min. 40 µl PBS was added prior to the subsequent dilutions which were used in the dot immunobinding assay.

A17 DETERGENTS.

Sodium dodecylsulphate (SDS) (lauryl sulphate, sodium salt).
Nonidet P40 (NP 40).
Triton X-100.
Tween 20 (T).

These were prepared in PBS at a concentration of 3%, 1%, 0.3%, 0.1% and 0.01% for use as the sample extraction buffer.

A18 BOILING MIX.

All reagents were prepared from electrophoresis grade chemicals.

18.1 Boiling mix.

Stacking gel buffer	1.0 ml
25% (w/v) SDS	0.8 ml
β-mercaptoethanol	0.5 ml
Glycerol	1.0 ml
Bromophenol blue	added for colour

18.2 Stacking gel buffer.
Tris (5.9 g) and SDS (0.4 g) were dissolved in 80 ml u-p water, the pH was adjusted to 6.7

using concentrated HCl. U-p water was added to a final volume of 100 ml.

A19 GLASS BEADS - BDH, 40 mesh.

The required concentration of fungal mycelium in 1 ml PBS was added to 0.75 g glass beads in an eppendorf tube and was vortexed for 5 x 1 min periods with intervening 1 min periods at 4°C. A hole, whose diameter was smaller than the beads, was made in the base of the eppendorf tube and it was inserted into the punctured lid of a larger tube. These tubes were centrifuged at 3000 rpm for 10 min and led to separation of the protein solution from the beads. The supernatant protein solution was removed and, prior to storage at -20°C, boiling mix was added in the proportion of 2 parts supernatant protein sample: 1 part boiling mix.

A20 PREPARATION OF SAMPLES FOR ELECTROPHORESIS.

\+ means stage included in protocol.

Preparation variants	Preparation temperature		Pre-grind addition			Grind	Post-grind treatment						
	R/T	4°C	PBS	Boiling mix	PMSF solution		Wait R/T	Wait 4°C	Boiling mix	100°C 3 min	Wait R/T	Wait 4°C	Storage at -20°C
Preparation method													
1	+		+			+	+		+		+		+
2		+	+			+		+	+			+	+
3	+		+	+		+	+				+		+
4		+	+	+		+		+				+	+
5	+		+	+		+	+			+			+
6		+	+	+		+		+		+			+
7	+				+	+			+		+		+
8		+			+	+			+			+	+
9	+				+	+			+	+			+
10		+			+	+			+	+			+

A21 PHENYLMETHYL-SULPHONYL FLUORIDE (PMSF).

A 1.2 mg/ml solution of PMSF in PBS was prepared by initially dissolving 30 mg PMSF in 1 ml dimethyl sulphoxide (DMSO) with a subsequent dilution by PBS. The solution was kept at 4°C until used as the sample extraction buffer.

A22 COOMASSIE PROTEIN ASSAY REAGENT (Pierce).

0.5 ml protein solution and 0.5 ml Coomassie reagent were mixed and the absorbance at 595 nm was read after 2 min. The protein content of the fungal extract was determined by comparison with the BSA standard curve (A23).

A23 BOVINE SERUM ALBUMIN.

Albumin standard (BSA fraction V) (Pierce) was used at concentrations between 1 - 20 µg/ml in PBS to construct the standard curve.

A24 REAGENTS FOR SDS-PAGE.

All chemicals were electrophoresis reagent grade.

24.1 Gradient gel acrylamide.
Acrylamide (Fluka) (28.5 g) and N,N'-methylene-bis-acrylamide (1.5 g) were dissolved in 85 ml u-p water before being made up to 100 ml and filtered.

24.2 Stacking gel acrylamide.
Acrylamide (29.25 g) and bis-acrylamide (0.75 g) were dissolved in 85 ml u-p water before being made up to 100 ml and filtered.

24.3 Resolving gel buffer.
Tris (18.15 g) and SDS (0.4 g) were dissolved in 85 ml u-p water, the pH adjusted to 8.9 before being made up to a final volume of 100 ml.

24.4 Stacking gel buffer - see A18.2.

24.5 Ammonium persulphate.
10% (v/v) solution freshly prepared prior to use.

24.6 TEMED (N,N,N',N'-tetramethyl-ethylene diamine).

24.7 Butanol.
After pouring the resolving gel it was overlaid with butanol to prevent desiccation during polymerisation.

24.8 Glycerol.

24.9 Electrode buffer.
Tris (15.8 g), SDS (2.5 g) and glycine (10.0 g) were dissolved in 2.5 l u-p water. Storage was at 4°C prior to use.

24.10 Boiling mix - see A18.

A25 GEL MIXTURES.

The volumes given are for one 5-15% gradient gel run on a LKB 2001 Vertical electrophoresis unit.

		Resolving gel		Stacking gel
% gel concentration		5	15	
Reagent				
Gradient gel acrylamide	(ml)	2.7	8.0	-
Resolving gel buffer	(ml)	4.0	4.0	-
Stacking gel acrylamide	(ml)	-	-	2.0
Stacking gel buffer	(ml)	-	-	3.0
U-p water	(ml)	9.3	1.6	7.0
Glycerol	(ml)	-	2.4	_
Ammonium persulphate	(µl)	100	43.3	100
TEMED	(µl)	6.6	6.6	5.0

A26 STANDARD PROTEINS.

Standard proteins, low molecular weight (LMW) and high molecular weight (HMW), were initially prepared according to the manufacturer's instructions and stored at -20°C in 100 µl aliquots. Prior to use they were diluted 1:10 in PBS:boiling mix (2:1) and heated at 100°C for 1 min.

1. LMW standard protein solution (SDS-7) included myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine albumin (66

kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

2. HMW standard protein solution (SDS-6H) included bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa).

A27 SILVER STAINING REAGENTS.

All reagents were made with analar grade chemicals. Pretreat, impregnate and develop were prepared immediately prior to use.

27.1 Fix.
50% (v/v) methanol and 12% (v/v) acetic acid containing 0.5 ml of 37% formaldehyde solution (Fisons)/l.

27.2 Wash A - 50% ethanol (v/v).

27.3 Pretreat.
0.02% (w/v) sodium thiosulphate.$5H_2O$ (Fisons).

27.4 Impregnate.
0.2% (w/v) silver nitrate (anhydrous) (Fisons) containing 0.75 ml of 37% formaldehyde solution/l.

27.5 Develop.
6% (w/v) sodium carbonate (anhydrous) and 0.0004% (w/v) sodium thiosulphate.$5H_2O$ containing 0.5 ml 37% formaldehyde solution/l.

27.6 Stop.
50% methanol (v/v) in 12% (v/v) acetic acid.

27.7 Wash B - 50% (v/v) methanol.

27.8 Rinse and store - u-p water.

27.9 Pre-dry 1 - 30% (v/v) methanol.

27.10 Pre-dry 2 - 3% (v/v) glycerol.

A28 PROTEIN BLOTTING.

28.1 Buffers.

All buffers were stored at -20°C and used at 4°C.

1. Anode buffer 1.
   0.3M Tris-HCl, 20% (v/v) methanol, pH 10.4

   Tris-HCl (11.85 g) and analar methanol (50 ml) were diluted in 200 ml u-p water, pH adjusted to 10.4 and the volume made up to 250 ml.

2. Anode buffer 2.
   25mM Tris-HCl, 20% (v/v) methanol, pH 10.4

   Tris-HCl (0.9875 g) and analar methanol (50 ml) were diluted in 200 ml of u-p water, pH adjusted to 10.4 and the volume made up to 250 ml.

3. Cathode buffer.
   20mM Tris-HCl, 40mM amino-caproic acid, 20% (v/v) methanol, pH 9.4

   Tris-HCl (0.79 g), amino-caproic acid (1.312 g) and methanol (50 ml) were diluted in 200 ml u-p water, pH to 9.4 and made up to 250 ml.

28.2 Sartoblot apparatus.

Before use this was cooled to 4°C and the graphite electrodes wiped clean with u-p water.

The manufacturer's protocol was followed except that the blotting process was carried out at 0.8 mA/cm^2 of gel for 30 min at 4°C followed by 1.2 mA/cm^2 of gel for a further 30 min.

28.3 Chromatography paper.

Prior to assembly of the blotting 'sandwich' chromatography paper was cut into 6 sheets the size of the gel/membrane.

A29 INDIA INK SOLUTION.

Pelikan Fount India Drawing ink, No. 518 (black) at 0.0001% (v/v) in PBS/0.05%T.

A30 PROTEIN BLOTTING PARAMETERS.

Experimental parameters investigated in the improvement of definition of visualised antigens.

Parameter	Variation tested		
Membrane	Nitrocellulose, Immobilon		(A12)
Blotting conditions	Bio-Rad Trans-Blot	250 mA, o/night, 4°C. 92 mA, 3 h, 4°C.	
	Sartoblot II (A28)	1 mA/cm^2 gel, 60 min. 0.8 mA/cm^2 gel plus 1.2 mA/cm^2 gel - both 30 min, 4°C.	
Sample preparation	Grinding	Mortar/pestle. Glass beads	(A19)
	Diluent	PBS, boiling mix or PMSF solution	(A20)
	Temperature	4°C, R/T, 100°C	(A20)
Ag conc. for samples	6.25, 3.125, 1.56 and 0.78 mg/ml.		
Ab1	Dilutions	1:400, 1:800, 1:1600, 1:3200.	
	Incubation	60 min, overnight or 3 days.	
	Temperature	R/T or 4°C.	
HRP-Ab2	Dilutions	1:100, 1:200, 1:400.	
Chromogen	DAB, DAB/N		(A13)
Buffers	PBS plus NCS, T and B		(A11)

A31 GLYCAN DETECTION.

Reagents required in addition to those supplied with the Boehringer Glycan Detection kit (A10).

31.1 Tris buffered saline (TBS), pH 7.5
0.05M Tris-HCl containing NaCl (0.15M) was adjusted to a pH of 7.5 prior to final volume.

31.2 Sodium acetate buffer 0.01M, pH 5.5
0.01M sodium acetate was adjusted to pH 5.5 before being made up to final volume.

31.3 Tris buffer, pH 9.5

0.1M Tris-HCl plus $MgCl_2$ (0.05M) and NaCl (0.1M) was adjusted to pH 9.5 prior to final volume.

31.4 Dimethyl formamide.

31.5 PBS, pH 6.5
0.05M potassium phosphate plus NaCl (0.15M) was adjusted to pH 6.5 prior to final volume.

A32 LECTINS FOR GLYCAN DETECTION.

32.1 Peroxidase labelled lectins.

1. Concanavalin A (Con A) from Canavalia ensiformis.
2. Wheat germ agglutinin (WGA) from Triticum vulgaris.

These were reconstituted according to the manufacturer's instructions and stored in 25 µl aliquots at -20°C.

32.2 Diluents and buffers for peroxidase labelled lectins.

	Con A assay	WGA assay
Diluent for lyophilised lectin	PBS 0.01M, pH 6.8 with $CaCl_2$ 0.1mM and $MnCl_2$ 0.01mM	PBS 0.01M, pH 6.8
Concentration of lectin in assay	2.5 µg/µl	20 µg/µl

Buffers.

Block	Tris/0.5T/saline	PBS/0.5T
Dilution	Tris/0.05T/saline	PBS/0.05T
Wash	Tris/0.05T/saline	PBS/0.05T

32.3 Tris/T/saline buffer, pH 7.5
0.01M Tris-HCl was adjusted to pH 7.5, after which NaCl (0.15M), $CaCl_2$ (0.1mM) and $MnCl_2$ (0.01mM) were added. T was subsequently added at a concentration of either 0.5% or 0.05% (v/v).

APPENDIX B.

PUBLICATIONS.

Palfreyman, J.W., Bruce, A., Button, D., Glancy, H., Vigrow, A. & King, B. (1987). Immunological methods for the detection and characterisation of wood decay Basidiomycetes. In Biodeterioration 7 (eds D.R. Houghton, R.N. Smith & H.O.W. Eggins), pp 709-713. London, UK: Elsevier.

Palfreyman, J.W., Vigrow, A., Button, D. & Glancy, H. (1988). Simple method for scanning immunoblots. Journal of Immunological Methods 109, 199-201.

Palfreyman, J.W., Glancy, H., Button, D., Vigrow, A., Score, A. & King, B. (1988). Use of immunoblotting for the analysis of wood decay Basidiomycetes. International Research Group on Wood Preservation, Document No: IRG/WP 2307.

Vigrow, A., Button, D., Palfreyman, J.W., King, B. & Hegarty, B. (1989). Molecular studies on isolates of Serpula lacrymans. International Research Group on Wood Preservation, Document No: IRG/WP 1421.

Palfreyman, J.W., Vigrow, A., Glancy, H. & King, B. (1990). Molecular studies on Serpula lacrymans. Paper presented (by J.W. Palfreyman) to the 4th International Mycological Congress, Regensburg, Germany.

Palfreyman, J.W., Vigrow, A., Glancy, H. & King, B. (1990). Molecular studies on wood decay Basidiomycetes. Proceedings of the IVth COST-88 workshop on 'Methods for detecting and identifying plant pathogenic fungi', pp 48-49.

Vigrow, A., Glancy, H., Palfreyman, J.W. & King, B. (1991). The antigenic nature of Serpula lacrymans. International Research Group on Wood Preservation, Document No: IRG/WP 1492.

Palfreyman, J.W., Button, D., Glancy, H., King, B., Nicoll, G., Smith, G. & Vigrow, A. (1991). The detection and destruction of Basidiomycetes in the timber of artefacts of historical or archaeological interest. In Proceedings of the European Symposium on 'Science, Technology and European Cultural Heritage' (eds N.S. Baer, C. Sabbioni

& A.I. Sors), pp 642-645. Oxford, UK: Butterworth-Heinemann Ltd.

Palfreyman, J.W., Vigrow, A. & King, B. (1991). Molecular identification of fungi causing rot of building timbers. The Mycologist 5, 73-77.

Palfreyman, J.W., Vigrow, A., Button, D., Hegarty, B. & King, B. (1991). The use of molecular methods to identify wood decay organisms. 1. The electrophoretic analysis of Serpula lacrymans. Wood Protection 1, 15-22.

Vigrow, A., King, B. & Palfreyman, J.W. (1991). Studies of Serpula lacrymans mycelial antigens by Western blotting techniques. Mycological Research 95, 1423-1428.

Vigrow, A., Palfreyman, J.W. & King, B. (1991). On the identity of certain isolates of Serpula lacrymans. Holzforschung 45, 153-154.

Palfreyman, J.W. & Vigrow, A. (1992). Further molecular analyses of certain isolates of Serpula lacrymans (submitted for publication).

Vigrow, A. & Palfreyman, J.W. (1991). Immunological analysis of field samples of the dry rot organism Serpula lacrymans (manuscript in preparation).

The full text of the publications cited below has been removed from the e-thesis due to copyright restrictions:

Palfreyman, J.W., Bruce, A., Button, D., Glancy, H., Vigrow, A. & King, B. (1987). Immunological methods for the detection and characterisation of wood decay Basidiomycetes. In Biodeterioration 7 (eds D.R. Houghton, R.N. Smith & H.O.W. Eggins), 709-713. London, UK: Elsevier.

Palfreyman, J.W., Vigrow, A., Button, D. & Glancy, H. (1988). Simple method for scanning immunoblots. Journal of Immunological Methods 109, 199-201.

Palfreyman, J.W., Button, D., Glancy, H., King, B., Nicoll, G., Smith, G. & Vigrow, A. (1991). The detection and destruction of Basidiomycetes in the timber of artefacts of historical or archaeological interest. In Proceedings of the European Symposium on 'Science, Technology and European Cultural Heritage' (eds N.S. Baer, C. Sabbioni & A.I. Sors), 642-645. Oxford, UK: Butterworth - Heinemann Ltd.

Palfreyman, J.W., Vigrow, A. & King, B. (1991). Molecular identification of fungi causing rot of building timbers The Mycologist, 5, 73-77.

Palfreyman, J.W., Vigrow, A., Button, D., Hegarty, B. & King, B. (1991). The use of molecular methods to identify wood decay organisms. The electrophoretic analysis of Serpula lacrymans. Wood Protection 1, 15-22

Vigrow, A., King, B. & Palfreyman, J.W. (1991). Studies of Serpula lacrymans mycelial antigens by Western blotting techniques. Mycological Research 95, 1423-1428.

Vigrow, A., Palfreyman, J.W. & King, B. (1991). On the identity of certain isolates of Serpula lacrymans. Holzforschung 45, 153-154.

THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Document No: IRG/WP/2307

Working Group Biological Problems (Flora)

Use of Immunoblotting for the Analysis of Wood Decay Basidiomycetes

by

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Paper prepared for the Nineteenth Annual Meeting, Madrid, Spain
April 24-29 1988

IRG Secretariat
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Use of immunoblotting for the analysis of wood decay basidiomycetes

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Summary

Immunoblotting methods, in particular dot and Western blotting have been used to investigate features of a variety of wood decay organisms, in particular C.versicolor, L.lepideus and S.lacrymans. Antisera to each of these decay organisms has been produced by immunisation of rabbits with liquid culture grown hyphae. These antisera, after appropriate preabsorption with sawdust have been used to detect decay organisms grown in their natural substrate, i.e. wood.

Production of a semi-quantitative assay for C.versicolor allowed the relationship of antigen content to weight loss to be investigated in wood block experiments. Uninfected blocks contained no detectable antigen. In infected blocks antigen could be detected, however antigen content appeared to be higher in extracts from blocks with low levels of weight loss compared to extracts from blocks with high weight loss.

Western blotting, designed to identify the antigenic species present in different cultures of C.versicolor, indicated that the antigenic nature of the organism depends upon its substrate and that during the decay process the nature of antigens produced by C.versicolor changed, i.e. antigens of different molecular weights were detected.

Application of the Western blotting technique to two strains of S.lacrymans indicated that they could easily be distinguished by their antigenic nature and this technique may have implications for fungal classification.

These investigations indicate that immunological methods have considerable potential for the detection of decay organisms and for the study of the decay process itself.

Key Words

Immunoblotting, wood decay basidiomycetes, antigenicity

Introduction

Rapid methods for the immunological detection and identification of wood decay basidiomycetes greatly enhance the range of studies which can be undertaken. Whilst it is currently possible to detect organisms by classical cultural studies such methods are time consuming and do not give an adequate representation of the microbial distribution within a wood sample.

A possible method for overcoming such limitations of is the use of immunologically based probes. The development of immunological probes relies upon the ability of higher animals to produce specific antibodies against foreign materials (antigens) encountered by their immune systems. These antibodies, present in serum and constituting the active component of an antiserum, will react with antigen both in the animal and in in vitro tests. By labelling, or tagging the antibody in some way, for example with an enzyme or a fluorescent dye, it is possible to visualise the reaction of an antibody with its antigen in an in vitro test. Such tests, which form the basis of the set of techniques termed 'Immunotechnology', can be used to assay an antigen or the organism from which it comes, to detect antigen microscopically (at the light or electron microscope level), to analyse the molecular nature of an antigen, to study its development and metabolism, or a wide range of other features of a specific antigen. Whilst antibodies are usually produced against animal pathogens almost any molecule can be induced to produce an antibodies (and hence an antiserum) within an experimental animal given suitable protocols.

Despite the wide use of immunotechnology in various fields of biology its application to the study of wood decay basidiomycetes has only recently been investigated (Goodell and Jellison 1986, Jellison and Goodell 1986, Palfreyman et al 1987). However the large range of available techniques indicate the potential that this technology has in the study of both the wood decay basidiomycetes and the wood decay process itself.

This paper summarises the initial results of studies made with antisera to a variety of decay organisms, in particular C.versicolor, L.lepideus and S.lacrymans. Two types of immunological technique have been utilised in this present study, viz. immuno-dot blotting and Western blotting. Both of these techniques utilise the ability of nitrocellulose membranes to bind antigens and the subsequent ability of antibodies to detect bound antigens. In the dot blotting technique simple presence or absence of an antibody/ antigen reaction is detected, in the Western blot antigens are separated electrophoretically prior to blotting and the relative molecular weights of antigens can be estimated.

Materials and Methods

The organisms used in this study, Coriolus versicolor (L ex Fr) Quelet (FPRL 28A), Stereum sanguinolentum (Alb & Schwein ex Fr) Fr (FPRL 27D), Lentinus lepideus (Fr ex Fr) Fr (FPRL 7F), Gloeophyllum trabeum (BAM(EDW)109), G.sepiarium (Wulfen ex Fr) Karsten (FPRL 10D), Schizophyllum commune Fr (FPRL 9), and Serpula lacrymans (Schumacher ex Fr) Gray CMI 79 125 (FPRL 12C) and CMI 152 233, were supplied by Dr A.Bravery of the Building Research Laboratory, Princes Risborough, U.K..

All antigens used in this study were prepared from liquid culture grown organisms, harvested and washed briefly in deionised water, freeze dried and stored at -20oC until use. Such preparations were ground up in phosphate buffered saline (PBS) and a supernatants prepared for dot blot assay and sodium dodecyl sulphate (SDS) extracts for Western blotting. Antisera used in these studies were raised in rabbits by multiple subcutaneous injections PBS extracts emulsified in Freunds complete adjuvant. Approximately 2ml of immunogen was injected into each animal corresponding to 10mg of antigen. After booster immunisations with antigen in incomplete adjuvant animals were bled regularly at approximately fortnightly intervals.

Antisera development and the detection of antigens using these antisera were by the immuno-dot blot method which will be described in detail elsewhere (Glancy et al manuscript in preparation). Briefly, antigens from the appropriate organism, viz. either an organism against which antiserum had been raised or an one which was being tested for cross reactivity, were extracted as described above in PBS and then absorbed onto deionised water washed nitrocellulose strips followed by air drying. After blocking of excess antigen binding sites by incubation of the strips in PBS containing 0.5% Tween, 5% newborn calf serum for 60 min at room temperature, the strips were incubated with appropriately diluted (in PBS containing 0.05% Tween, 5% newborn calf serum) antiserum at room temperature for 60 min. After 6 washes with PBS containing 0.05% Tween the strips were incubated with diluted peroxidase linked antirabbit serum (from the Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.) for a further 60 min at room temperature then washed and incubated with the peroxidase substrate diamino- benzidine and hydrogen peroxide.

Western blotting was essentially by the method of Towbin and Gordon (1984) with modifications which will be described in detail elsewhere (Glancy et al manuscript in preparation). Briefly samples were extracted into boiling mix (Towbin and Gordon 1984), electrophoresed on 7.5% SDS polyacrylamide gels, electroblotted onto nitrocellulose then detected using appropriately diluted antiserum followed by peroxidase linked antirabbit serum. Antibody binding was again visualised by diamino-benzidine.

When assaying wood samples for antigen, blocks were ground up to a fine sawdust and antigens extracted into PBS. All antisera used for detecting wood grown hyphae were preincubated with sawdust (2mg/ml)

prior to use to reduce non-specific reactions between wood and antisera. Where appropriate, antisera were preincubated with hyphal extracts, 2mg/ml, to remove cross reacting antibodies. Both types of preincubation were carried out for 60 min at room temperature.

Semiquantitive analysis of dot blot immunoassays was achieved by rendering the nitrocellulose transparent with xylene followed by scanning densitometry with an LKB laser densitometer. A scan which relates peak height to intensity of the immuno-dot is produced from which a semi-quantitative analysis may be made (Palfreyman et al 1988).

Results and Discussion

Antisera against all three organisms studied reacted strongly in the immuno-dot blot assay with the appropriate antigen. Cross reactivity with other fungi was noted for each of the antisera, little such reactivity, however, was found with non-basidiomycetes (Glancy et al manuscript in preparation). Antisera cross reactivity with inappropriate basidiomycetes, i.e. not the immunising organism, could be markedly reduced by preabsorption of antiserum with antigen. See Table 1 for C.versicolor. Thus although the C.versicolor antiserum reacted with S.sanguinolentum, S.commune, L.lepideus, G.sepiarium and G.trabeum this cross reactivity could be effectively removed by preincubation with L.lepideus and S.sanguinolentum. Different combinations of organisms could render the other antisera organism specific.

TABLE 1

Effect of preabsorption of C.versicolor antiserum on the reaction with various basidiomycetes in the immunodot blot method.

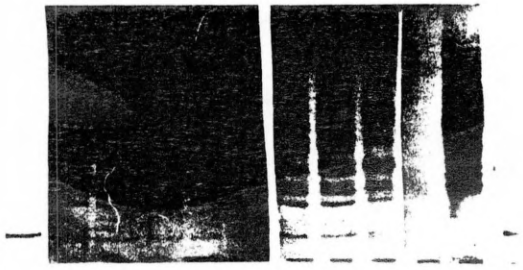
C.versicolor antiserum was preincubated with extracts of L.lepideus and S.sanguinolentum. The preabsorbed antiserum was then tested for its reaction against a number of other basidiomycete organisms. The reaction of the antiserum with hyphal extracts is rated from ++++ to -.

Organism	Unabsorbed	Absorbed with L.lepideus and S.sanguinolentum
C.versicolor	+++	+++
S.sanguinolentum	+++	-
S.commune	+++	-
L.lepideus	+++	-
G.sepiarium	+++	-
G.trabeum	++++	+

The apparent changes in antigen concentration found at different weight losses indicated either that there were similar changes in hyphal mass within the wood blocks, or that further changes in antigenic nature were occuring during the later stages of decay. Western blotting of extracts from decayed blocks was used to test the validity of these hypotheses (Fig 1). A liquid culture grown hyphal extact (track 2) is compared in this figure with extracts from blocks showing low weight loss (i.e weight loss of <10%, tracks 7-12) and those showing high weight loss (i.e. weight loss >10%, track 3-6). Considerable differences in the antigens detected in these various tracks are apparent.

Fig. 1. Western blots of C.versicolor decayed wood blocks.

Tracks 1 and 13 molecular weight markers, track 2 an extract of liquid culture grown hyphae, tracks 3-6 infected wood blocks showing high weight loss (>10%), tracks 7-12 infected blocks showing <10% weight loss.



The results shown in Fig 1 illustrate that different antigenic species are present at different stages of decay. They also demonstrate the the potential of the Western blotting technique for studying the antigenic nature, and hence the molecular structure, of wood decay fungi. To test if this method could also be used as an aid to classification, Western blotting of two strains of the decay fungi S.lacrymans was carried out using an antiserum developed against this basidiomycete.

The results of this experiment are shown in Table 3. At least 13 antigens were detected in S.lacrymans strain FPRL 12C and 12 antigens in strain CMI 152 233. The actual molecular weights of the antigens identified are reported in Table 3.

TABLE 3

Antigens of S.lacrymans strains detected by Western blotting.

S.lacrymans	FPRL 12C	CMI 152 233
	162	162
molecular	120	120
weight	100	100
of major	87	
antigens	76	76
	64.5	64.5
(x10)	57.5	57.5
	47	47
	31.5	31.5
	22.5	22.5
	19	
	13.5	13.5
	9	9
		8

Antigens from two strains of S.lacrymans were separated electrophoretically and detected with the S.lacrymans antiserum by the Western blotting technique. The molecular weight of the antigens detected was determined by comparing the relative mobility of antigen bands with the mobility of standard molecular weight marker proteins.

From these results it can be seen that two antigens (mol wt 87,000 and 19,000) present in FPRL 12C are absent in 152 233, and one antigen (mol wt 8,000) is present in the latter and is absent from FPRL 12C.

Conclusions

The results of this study indicate that sensitive and specific immunoblotting assays against wood decay fungi can be developed with reagents produced against hyphal extracts. These assays, and associated techniques can be used to study organisms during the decay process and indicate that the antigenic, and hence molecular, structure of these organisms changes during decay. Our results have been obtained with polyclonal antisera, the development of monoclonal antibody based reagents in the future can only enhance the value of immunological methods to the study of wood decay.

References

Goodell,B.S., and Jellison,J. (1986). Detection of a brown-rot fungus using serological assays. Document No: IRG/WP/1305

Jellison,J. and Goodell,B.S. (1986). Production of monoclonal antibodies to fungal metabolites. Document No: IRG/WP/1306

Palfreyman,J.W., Bruce,A. Button,D., Glancy,H., Vigrow,A. and King,B. (1987). Immunological methods for the detection and characterisation of wood decay basidiomycetes. Proceedings VIIth Int. Biodeterioration Symposium (in press).

Palfreyman,J.W., Vigrow,A., Button,D. and Glancy,H. (1988) A simple method for scanning immunoblots. J.Immunol. Methods (in press)

Towbin,H. and Gordon,J. (1984). Immunoblotting and dot blotting - current status and outlook. J.Immunol. Methods 72, 313-340.

Document No: IRG/WP/1421

THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Working Group I a Biologiacal Problems (Flora)

Molecular studies on isolates of *Serpula lacrymans*

by

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Paper prepared for the 20th Annual Meeting
Lappeenranta, Finland
22-26 May 1989

IRG Secretariat
Box 5607
S-114 86 Stockholm
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22 May 1989

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Summary

The major protein species present in detergent extracts of 14 different Serpula lacrymans isolates have been compared, by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), with a standard strain, viz. S.lacrymans FPRL 12C. Following silver staining of SDS gels the major protein species identified in 12 isolates were similar to those found in the standard strain. However differences were found when the final two isolates were compared with FPRL 12C, both isolates had extra molecular species not present in 12C and both were lacking some present in the standard strain.

Comparison of the protein species identified in S.lacrymans isolates with those identified in extracts of other fungal organisms, viz. brown and white rot causing basidiomycetes and non-basidiomycetes indicated that the Serpula isolates were more similar to each other than to other organisms. Some molecular differences could be identified when individual isolates were cultured on different media, i.e. liquid culture or agar, only minor differences were seen when individual isolates were subcultured.

These results indicate that whilst care must be taken to ensure as near identical conditions as possible for culture of organisms if their molecular species are to be compared by SDS-PAGE and silver staining, consistent results can be obtained using this technique. The technique may therefore offer a method of distinguishing between isolates, strains and species of wood decay basidiomycetes, and identifying new isolates.

Keywords: molecular analyses, Serpula lacrymans, SDS-PAGE.

Introduction

There are numerous reports on the use of electrophoretic patterns of soluble proteins extracted from fungal mycelia to aid in the identification and classification of fungal isolates (Clare, 1963, Bent, 1967, Milton et al, 1971). In particular the technique of SDS-PAGE has been used to distinguish between species of Sclerotinia (Tariq et al 1985), to identify aggressive and non-aggressive strains of Ceratocystis ulmi (Jeng and Hubbes, 1983) and to differentiate strains of yeast on the basis of their excreted macromolecules (Bouix and Leveau, 1983). More recently identification of different species of Phytophthora, a major plant pathogen has been achieved by comparison of electrophoretic profiles (Hansen et al, 1986, Hansen et al 1988). Application of similar molecular methods to the identification and classification of basidiomycetes causing structural decay of timber, in particular S.lacrymans, is the basis of the studies reported in this paper.

S.lacrymans has been isolated from infected timber from a number of areas of the world including Europe and Australasia (Cymorek and Hegarty, 1986). Comparison of isolates from such diverse areas demonstrates a range of morphological and growth characteristics which are not obviously related to geographical origin (Cymorek and Hegarty, 1986). In order to investigate the possible molecular basis of differences between S.lacrymans isolates, to aid in the identification of S.lacrymans and to distinguish it readily from other basidiomycetes, such as Fibrioporia vaillantii which cause damage similar to that produced by S.lacrymans (Bravery et al, 1987), we have initiated an electrophoretic study of detergent soluble proteins produced by a range of S.lacrymans isolates. The molecular species present in S.lacrymans are also compared with those extracted from other fungal species.

Material and Methods

S.lacrymans strain FPRL 12C was supplied by Dr A.R.Bravery of the Building Research Establishment (B.R.E), Garston, U.K. and strain CMI 152233 by the Commonwealth Mycological Institute, Kew, London, U.K.. Other isolates were collected from various sites around the world by one of the authors (B.M.H.). The designations and geographical origin, where known, of the isolates used in this study are given in Table 1.

Table 1

List of the S.lacrymans isolates and preparations used in this study.

	Strain (culture)	Original code	Isolation Date	Source
1	FPRL 12C			UK
3	CMI 152233			UK
4	BF-01		1965	France
5	BF-03A	Ebw.315	1936	Germany
6	BF-07B	A-169	1967	UK
7	BF-015B			GDR
8	BF-017B	BAM 133G	1937	France
9	BF-018A	BAM 238	1939	Germany
10	BF-023		1984	GDR
11	BF-025		1984	GDR
12	BF-044	FP 90876-R	1946	USA
13	BF-046	Warsaw III		CSIRO
14	BF-049	DFP 16521	1981	Australia
15	BF-050	DPF 16522	1981	Australia
16	BF-072		1930's	Germany

A number of the other fungal species used in this study were obtained from B.R.E.. These organisms are listed in table 2.

List of fungal species obtained from B.R.E. and used in this study

	Species	Strain
1	Coniophora puteana	FPRL 11E
2	Fibrioporia vaillantii	FPRL 14G
3	Postia placenta	FPRL 280
4	Gloeophyllum sepiarium	FPRL 10D
5	Lentinus lepideus	FPRL 7F
6	Stereum sanguinolentum	FPRL 27D
7	Heterobasidion annosum	FPRL 41E
8	Schizophyllum commune	FPRL 9
9	Pleurotus ostreatus	FPRL 40A

Also used in this study were Gloeophyllum trabeum BAM(EDW) 109, Cladosporium resinae BM 13388-1-22A, Paecilomyces variotti (isolated at Dundee Institute of Technology) and Trichoderma polysporium IMI 206039.

Preparation of mycelial extracts

For preparation of extracts for electrophoretic analysis S.lacrymans isolates were grown in approximately 20ml of 5% malt extract broth in petri dishes, incubated at 22oC and harvested when 75% of the liquid medium surface was covered in mycelium. Harvesting consisted of removal of original core followed by washing of the remaining mycelium with ultra-pure water to remove medium components (3 washes). The remaining material was partially dried on a filter paper. This material was then freeze dried and stored at -20oC until analysis. Other fungal microorganisms, i.e. other than the Serpula isolates , were prepared in a similar manner.1

Prior to electrophoresis weighed samples of freeze dried material were ground up in phosphate buffered saline pH 7.4 (PBS) (6.25mg mycelium: 1ml buffer) using a pestle and mortar. Once a fine slurry had been produced, 1 part boiling mix (Marsden et al (1978)) was added to 2 parts sample. The resulting mix was stored at -20oC until use. Before adding samples to gels they were thawed, heated to 100oC for 3 min and centrifuged at 13,000 rpm for 10 min at room temperature. 20ul samples of supernatant were used for analysis by SDS-PAGE. To test the effect of substrate on the molecular nature of S.lacrymans the organism was grown on 5% malt extract/ 2% agar until 75% of the medium surface was covered in mycelium. The original core was cut out and the remaining mycelium was scraped off the agar. This material was freeze dried and stored at -20oC until analysis. Subsequent preparation was similar to that of liquid culture material.

Electrophoresis

Mycelial extracts were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970) as modified by Marsden et al (1978). Briefly samples prepared as above were applied to the wells of a 5-15% gradient gel prepared using ultrapure water as described by Marsden et al (1978) using ultra-pure water to allow subsequent silver staining of the gel. To allow comparison of the standard isolate of S.lacrymans used in this analysis (FPRL 12C) with test strains, samples were

loaded on to gels as illustrated in Fig. 2, i.e. FPRL 12C was placed in alternate tracks with test isolates intervening. Samples were then electrophoresed for 4 hours at 35mA/ gel at 4oC on a LKB 2001 vertical electrophoresis unit.

Silver staining

All solutions used in the silver staining procedure were made up in ultrapure water as were solutions used in gel preparation and sample extraction. After electrophoresis gels were fixed overnight with a solution of methanol/acetic acid:formaldehyde (50%:12% + 0.5ml 37% HCOH/L) and stained according to the method of Blum et al (1987). In this method gels are pretreated with sodium thiosulphate, impregnated with silver nitrate and finally developed with a solution containing sodium carbonate/ formaldehyde and sodium thiosulphate.

Analysis of gel patterns

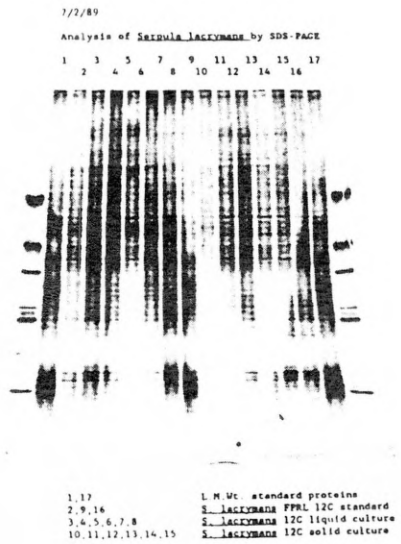
Following silver staining maps were constructed of the protein pattern produced for S.lacrymans FPRL 12C based on a visual survey of 6 repeated analyses of S.lacrymans. The staining intensity of each protein band was visually evaluated and recorded on a scale of 1 to 3. Similar maps were constructed for other isolates to allow comparisons of banding patterns. To aid in the comparison of protein bands from different isolates bands were numbered according to their position in the gels and approximate molecular weights of the proteins represented by specific bands were determined.

Results

Preliminary experiments were carried out to investigate whether or not cultures of S.lacrymans FPRL 12 gave consistent banding patterns on the gel system utilised. Results indicated that during passage of the organism the staining pattern remained essentially the same though if the organism was grown in different culture systems, e.g. on agar rather than in liquid culture, some minor changes in banding patterns were observed, (see Fig. 1).

Fig. 1.

Analysis of the major protein species found during culture of S.lacrymans FPRL 12C



Tracks 1 and 17 represent standard molecular weight markers used in these experiments, reading from the top of the gel the bands correspond to proteins of molecular weight 66,000, 45,000, 36,000, 29,000, 24,000, 20,100 and 14,200 daltons respectively. Tracks 2, 9 and 16 represent extracts of the standard preparation of S.lacrymans FPRL 12C used throughout this study as a reference. Tracks 3,4,5 and 6 represent extracts of liquid cultures of FPRL 12C harvested after 3, 5, 7 and 10 days after the first subculture. Tracks 7 and 8 represent the 10 day old harvests of subcultures 2 and 3.

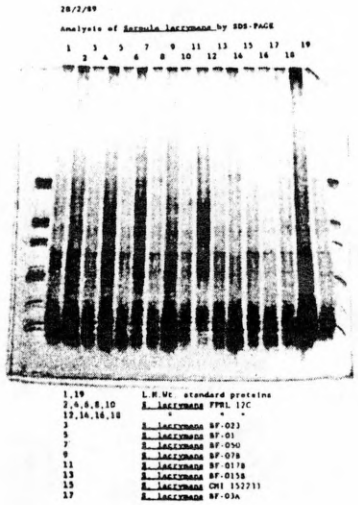
Tracks 10,11,12 and 13 represent extracts of 3,5,7 and 10 day old cultures grown on solid medium, tracks 14 and 15 represent 10 day old harvests of solid culture grown material, subcultures 2 and 3.

Comparison of the 14 isolates listed above with the standard isolate used in these studies (FPRL 12C) indicated that the majority of bands were common between 12C and the others (Fig. 2). Two of the S.lacrymans isolates however, BF-015B and BF-050 produced unusual banding patterns though it can be seen that the majority of the protein species detected in extracts of these two

isolates are similar to those of FPRL 12C. Notable new bands and missing bands are listed in Table 3. Comparison of the data shown in Figs. 1 and 2 further illustrates that the banding patterns obtained for FPRL 12C during these electrophoresis experiments were reproducible.

Fig 2.

Analysis of the major protein species found in a selection of isolates of S.lacrymans. Tracks are numbered from the left and the key to the identification of tracks is shown in the figure.



The data illustrated in Figs. 2 indicates that there is some variability in the banding pattern produced by individual isolates of S.lacrymans. To compare the variability obtained within a species with that obtained between species SDS-PAGE analysis was carried out on a variety of other brown and white rots and other, non-decay organisms. A selection of the results of this experiment are shown in Fig. 3. None of the fungal extracts tested showed banding patterns similar to S.lacrymans FPRL 12C.

Table 3

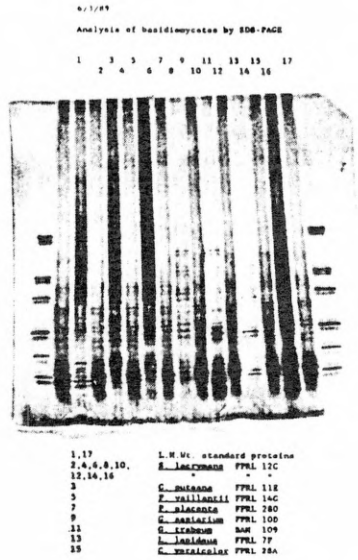
Major differences between S.lacrymans FPRL 12C, BF-050 and BF-015B.

Band Number	12C	BF-050	BF-015B	MWt (kD)
4.1	-	-	+	78
4.2	-	-	+	62
5.1	-	-	+	59
5.2	+	+	-	58
5.35	+	+	-	55
5.55	+	+	-	52
7.05	-	+	-	34
7.35	+	-	-	32
7.4	-	+	-	31.5
8.45	-	+	-	24
8.6	-	+	-	23
8.7	+	-	-	22.5
10.05	+	+	-	15.5

In this table band number is an arbitrary designation relating bands to their position in the gel.

Fig.3.

Analysis of the major protein species identified in a selection of brown rot basidiomycetes.

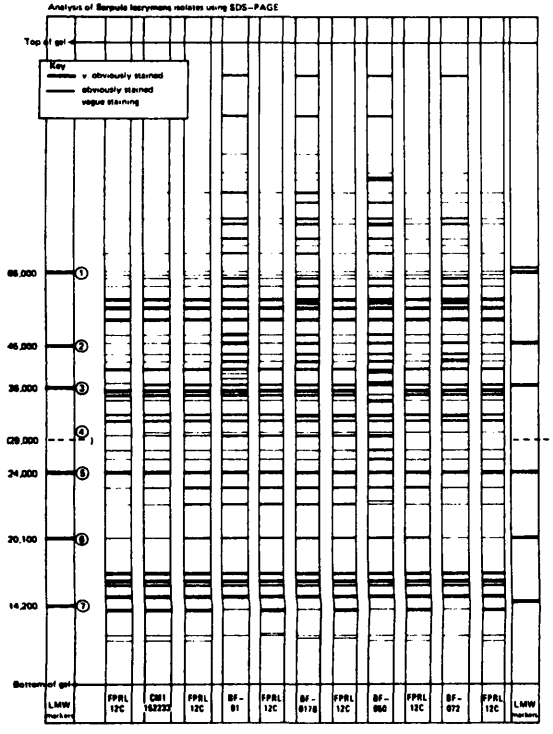


Only a selection of the organisms tested in this study (see Material and Methods) are shown in Fig. 3. However similar results were found for the other organisms listed in Table 2, viz. the molecular species identified in fungi other than S.lacrymans are very different than those forming the basic banding pattern of S.lacrymans.

Photographic representations shown in this paper do not reveal all bands detectable by eye and furthermore the staining patterns of the gels fades slowly. To aid in the analysis of the banding patterns shown in Figs. 1-3 representative diagrams have been produced. An example of such a diagram is shown in Fig. 4. Some of the differences between FPRL 12C and BF-050 are well shown in this diagram.

Fig 4.

Representation of an SDS-PAGE experiment. Bands are represented on a scale of 1 to 3, i.e. from very obviously stained to vague staining.



The data shown in Fig. 4 include representation of all the bands found in an individual experiment. A selection of those bands represented for FPRL 12C have been used as the basis of the comparison of isolates reported in this study.

Discussion

Electrophoretic techniques have been used extensively for the analysis of fungal isolates and can provide information on the affinities of species and variation between species. The initial objective of this present study was to determine if isolates of S.lacrymans obtained from various parts of the world would demonstrate similar protein profiles in SDS-PAGE. Isolates were compared with a standard strain of S.lacrymans (FPRL 12C) which itself gave consistent electrophoretic patterns during numerous subcultures. Of the 14 isolates compared with 12C only 2 showed any marked differences. As yet no obvious relationships between these 2 isolates and any morphological features of the isolates, their place of isolation or their growth rate has been established.

Though subculture of 12C had little effect on the molecular species detected in this analysis (Fig. 1) some minor differences were found when the banding patterns obtained from liquid culture extracts were compared with extracts of agar grown material. Furthermore though not shown in this paper other evidence indicates that the molecular species detected in wood grown S.lacrymans are different again from the agar and liquid culture material. It is therefore important to compare cultures grown under conditions as identical as possible. Some features such as growth rate of different isolates however cannot be equalised, but as indicated above the banding patterns obtained in these studies did not correlate with growth rate.

Despite the marked differences in protein profiles for S.lacrymans BF-050 and BF-015B as compared to both FPRL 12C and the other 12 isolates studied the overall banding pattern for these two organisms were not dissimilar to the standard FPRL 12C pattern. However other fungal species gave very dissimilar protein profiles. These results indicate that SDS-PAGE offers a highly precise and relatively simple method for identifying new isolates.

Molecular differences found in this current study are mirrored by antigenic differences detected using Western blotting techniques (data not shown). Again relatively minor antigenic differences are found between isolates of S.lacrymans (Palfreyman et al 1988) whereas other fungal species give essentially different antigenic profiles.

In order to introduce a degree of statistical analysis to our studies we are currently investigating the possible application, to wood decay organisms, of a computer program produced for identifying affinities between Phytophthora species. (Crookes et al, 1985). Using this program it may be possible to produce information on the relatedness and hence divergence of individual isolates of S.lacrymans.

Though the combination of SDS-PAGE with silver staining is a highly sensitive technique allowing the detection of nanogram amounts of protein, the protein profiles shown in Figs 1-4 represent only a very minor sample of the proteins produced in a mycelial culture. As yet we have not been able to identify any molecular species which correlate with morphological or other biological properties of S.lacrymans. This may be due to the sensitivity and selectivity of the SDS-PAGE system used. It may therefore be appropriate to investigate the potential of other molecular techniques such as two-dimensional electrophoresis or isoelectric focussing in the analysis of fungal isolates. Alternatively use of specific molecular probes, such as antibodies, may produce information relating biological properties to molecular components.

ine studies reported in this paper together with other analyses using Western blotting currently underway will allow information on the molecular relationships between various isolates S.lacrymans to be identified and, perhaps, allow the development of a molecular basis for the differentiation of basidiomycete isolates, strains and species.

References

Bent, K.J., 1967. Electrophoresis of proteins of 3 penicillium species on acrylamide gels. J. gen. Microbiol, 49 195-200.

Blum, H., H. Beier and H. J. Gross, 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis, 8 93-99.

Bouix, M. and J.Y. Leveau, 1983. Electrophoretic study of the macromolecular compounds excreted by yeasts, application to differentiation between strains of the same species of Saccharomyces cerevisiae. Biotechnol. Bioeng., 25 133-142.

Bravery, A.F., R.W. Berry, J.K. Carey and D.E. Cooper, 1987. In 'Recognising wood rot and insect damage in buildings'. Buildings Research Establishment Report, Building Research Establishment, Bucks, U.K..

Clare, B.G., 1963. Starch-gel electrophoresis of proteins as an aid in identifying fungi. Nature, 200 803-804.

Crookes, D., I. Morris, G.C. O'Doherty and A.R. McCracken, 1985. Matching electrophoretic patterns: a case study in computerisation. Software & Microsystems, 4 40-44.

Cymorek, S. and B. Hegarty, 1986. Differences among growth and decay capacities of 25 old and new strains of the dry-rot fungus Serpula lacrymans using a special test arrangement. Material und Organismen, 21 237-249.

Hansen, E.M., C.M. Brasier, D.S. Shaw and P.B. Hamm, 1986. The taxonomic structure of Phytophthora megasperma: evidence for emerging biological species groups. Trans. Br. mycol. Soc., 87 557-573.

Hansen, E.M., P.B. Hamm, C.G. Shaw III and P.E. Hennon, 1988. Phytophthora drechsleri in remote areas of southeast Alaska. Trans. Br. mycol. Soc., 91 379-384.

Jeng, R.S. and M. Hubbes, 1983. Identification of aggressive and nonaggressive strains of Ceratocystis ulmi by polyacrylamide gradient gel electrophoresis of intra mycelial proteins. Mycotaxon, 17 445-455.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature, 227 680-685.

Marsden, H.S., N.D.Stow, V.G.Preston, M.C.Timbury and N.M.Wilkie, 1978. Physical mapping of herpes simplex virus induced polypeptides. J. Virol, 28 624-642.

Milton, J.M., W.G. Rogers and I. Isaac, 1971. Application of acrylamide gel electrophoresis of soluble fungal proteins to taxonomy of Varticillium species. Trans. Br. mycol. Soc., 56 51-55.

Palfreyman, J.W., H. Glancy, D. Button, A. Vigrow, A. Score and B. King, 1988. Use of immunoblotting for the analysis of wood decay basidiomycetes. International Research Group on Wood Preservation Document Number IRG/WP 2307.

Tariq, V.N., C.S. Gutteridge and P. Jeffries, 1985. Comparative studies of cultural and biochemical characteristics used for distinguishing species within Sclerotinia.

Molecular Studies on *Serpula lacrymans*

by

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a paper prepared for

Workshop on *Serpula*

at

4th International Mycological Congress

Regensberg, August/September 1990

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INTRODUCTION

Research in biology in the last three decades has seen an exponential rise in the molecular analyses of organisms and processes. However the impact of the new knowledge and techniques associated with molecular biology, has only recently been apparent in research related to the dry rot fungus *Serpula lacrymans*. Indeed, in a very extensive bibliography compiled in 1988 (Seehann and Hegarty 1988) and which contains some 1,200 references to all aspects of *S.lacrymans* (its morphology, physiology, methods to control the organism, etc) there are few, if any, references to papers dealing with molecular aspects of the organism in terms normally associated with molecular biology (ie genetic studies at the DNA and RNA level and studies on the structure and function of proteins). The possible exception to this generalisation would be studies on the enzymes of *S.lacrymans* though such research is normally considered biochemistry rather than molecular biology.

It is in this context that the studies outlined in this paper were initiated, the intention being to apply techniques of molecular biology, and in particular those that research had shown to be relevant to other wood decay organisms (e.g. *Schizophyllum commune*, *Postia placenta* (*Poria placenta*) and *Lentinus lepideus* (de Vries *et al* 1980, Goodell and Jellison 1986, Palfreyman *et al* 1987)) to research on *S.lacrymans*. The techniques in question being electrophoresis and antibody based technology. The objective of the studies centered around developing reliable methods for the detection and identification of *S.lacrymans*. It was also hoped to further understanding of the physiology and metabolism of the organism to assist in the development, in the future, of more specific methods for its destruction and control.

IDENTIFICATION OF *S.Lacrymans*.

The identification of unknown isolates as *S.lacrymans* poses little problem to experts in the field of fungal taxonomy. The fruit body of the organism is easily distinguished from other basidiomycetes, with the possible exception of *S.himantioides* and in laboratory culture the organism shows a number of specific characteristics, not least amongst these being its unusual heat sensitivity and low temperature growth optimum. However identification of *S.lacrymans* by less experienced personnel is by no means so straightfoward and on occasion disputes arise, with major financial implications, around the identification of particular outbreaks of decay fungi. Since protein analysis has been used in numerous other areas as an aid in the identification of microorganisms (e.g. Tariq *et al* 1985, Jeng and Hubbes 1983, Hansen *et al* 1986) initial studies on *S.lacrymans* were designed to investigate the applicability of such types of analysis to this organism.

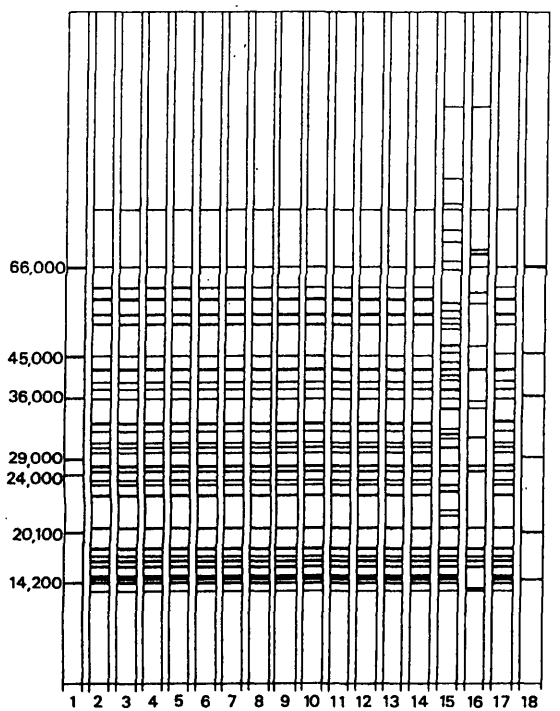
A group of thirteen isolates of *S.lacrymans* from Europe, the U.S.A. and Australia, were obtained from Dr B.Hegarty of Rohm and Haas, France and these are described in detail in Vigrow *et al* (1989). A further isolate, CMI 152233 was obtained from the Commonwealth Mycological Institute. Isolates were cultured using a standardised system (5% malt extract broth at 22oC), harvested and freeze dried. The dried material was then analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by the method

of Laemmli (1970) as modified by Marsden *et al* (1978). Separated proteins were visualised by the silver staining method of Blum *et al* (1987). Further details of the methodologies used can be found in Palfreyman *et al* (1990).

Initial studies were directed towards establishing if a consistent SDS-PAGE profile could be produced for a standard isolate of *S.lacrymans*. The standard isolate used in these studies being FPRL 12C obtained from the National Collection of Wood Rotting Fungi at the Building Research Establishment in the UK. A consistent banding profile was obtained for a series of extracts from different subcultures of this organism. Furthermore the growth of the organism on either solid or in liquid culture had no effect on the protein profile produced. A comparison of the range of 14 *S.lacrymans* isolates with the standard profile for FPRL 12C indicated that most of them were identical. However there were two isolates which had profiles which, whilst very similar to FPRL 12C, were different in a number of aspects (Fig 1, tracks 15 and 16). One of these organisms (Fig 1, track 16: BF-015B) has since been suggested to be, on the basis of its morphology, *S.himantioides* (Schmidt and Kebernik (1989)). Recent studies using SDS-PAGE and immunoblotting have confirmed this suggestion (Vigrow *et al* 1990).

Fig 1 Analysis of isolates of *S.lacrymans*

14 isolates of *S.lacrymans* (tracks 3-16) are compared with a standard preparation of FPRL 12C (tracks 2 and 17) in this figure. Tracks 1 and 18 represent molecular weight markers. Analysis of diagrams such as this has allowed the production of similarity indices (see table 1). The unusual profiles given by the two organisms mentioned in the text are shown in tracks 15 and 16.

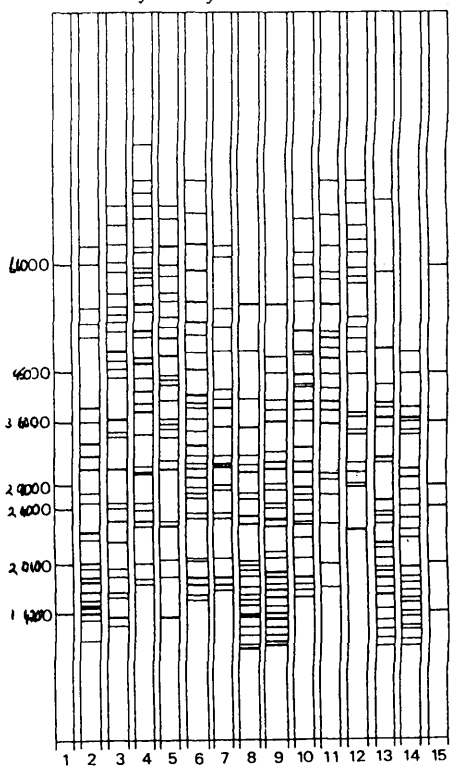


Whilst the profiles obtained for isolates of *S.lacrymans* and *S.himantioides* (e.g. Fig 1, tracks 16 and 17) are recognisably different it should be noted that they have more bands in common, and are therefore are more similar, than any other two species analysed to date. To determine whether members of other species which were formerly within the genus *Serpula*, *viz. S.tignicola* and *S.pinastri*, had profiles showing similarities to *S.lacrymans* and *S.himantioides* SDS-PAGE analysis was undertaken on extracts from freeze dried material of these organisms. Both of these organisms had profiles with very few similarities to those of either *S.lacrymans* or *S.himantioides*. It is interesting to observe that according to the most up to date information neither now belongs to the genus *Serpula*. Both organisms have now been reclassified into the genus *Leucogyrophana*. Molecular studies would appear to endorse this change.

If SDS-PAGE is to be used for identification purposes then it is essential that other organsisms that are likely to be found in a similar environment to *S.lacrymans* can be easily distinguished on the basis of their protein profiles. A number (18) of other wood decay basidiomycetes have now been analysed and a representation of the type of profile found is given in Fig 2. Three non-basidiomycetes have also been analysed by this method.

Fig 2 Comparison of SDS-PAGE profiles for *S.lacrymans* and other wood decay fungi.

Tracks in this figure represent, 1 and 15: mol. wt. markers, 2: *S.lacrymans*, 3: *Coniophora puteana*, 4: *Fibroporia vaillantii*, 5: *Paxillus panuoides*, 6: *Amylloporia xantha*, 7: *Leucogyrophana pinastri*, 8: *Lentinus lepideus*, 9: *Gloeophyllum sepiarium*, 10: *Peniophora gigantea*, 11: *Poria placenta*, 12: *Daedalea quercina*, 13: *Coriolus versicolor*, and 14: *Daldina concentrica*.



To use such data to evaluate relationships between organisms and to give a numerical value to the likelihood of identity between isolates it is probably

necessary to undertake the type of large scale study followed by complex computer analysis which was used by Kersters (1985) and Jackman (1985) for classification of bacteria. However to obtain some indication of relationships the current information has been analysed manually and a similarity index (SI) obtained which relates the number of bands an organism has in common with a reference organism (in most cases *S.lacrymans* FPRL 12C) and quotes the value as a percentage. Diagrams such as Figs 1 and 2 are used in this analysis. All isolates of *S.lacrymans*, except the two unusual ones noted above, gave similarity indices of >90%. The value obtained for *S.himantioides* is around 50%, whilst all other species tested had values of less than 31% (Table 1). One of the highest values was obtained for *Poria incrassata* which, interestingly, is often said to be the New World equivalent of *S.lacrymans* (Cartwright and Findlay 1958).

Table 1 Similarity indices for a selection of wood decay basidiomycetes

Organism	SI(%)	Organism	SI(%)
A.xantha	20.9	*P.gigantea*	10.7
C.puteana	18.5	*P.incrassata*	30.8
D.quercina	0.0	*P.placenta*	15.6
F.vaillantii	19.2	*P.panuoides*	21.3
G.sepiarium	19.8	*L.pinastri*	25.0
G.trabeum	24.0	*C.versicolor*	17.1
L.lepideus	16.0	*D.concentrica*	19.2

The above figures were derived from data of the type shown in Fig 2. Altogether in these studies 12 brown rot species (in addition to *S.lacrymans* and *S.himantioides*), white rot species and 3 miscellaneous species have been analysed by SDS-PAGE and an SI estimated. The mean values of the indices obtained for the three groups are 18.5, 19.6 and 19.2 respectively indicating that the similarity index cannot be used to distinguish between groups of fungi with different physiological characteristics. The results do, however, suggest that three specific types of organism can be distinguished by this method, *viz.* i) isolates of *S.lacrymans* (SI>90%), ii) isolates of *S.himantioides* (SI=50%) and iii) all other organisms tested to date (SI<31%).

These studies have indicated that the identification of *S.lacrymans* by SDS-PAGE is feasible and the technique has now been validated on a number of new isolates which give protein profiles very similar to FPRL 12C. Currently however it has only been confirmed that isolates of *S.lacrymans* grown up using a standard laboratory procedure can be identified by this method. Direct analysis of freeze dried field material, i.e. fruit bodies, strands, spores and surface mycelia, has indicated that different morphological forms of the fungus possess different protein profiles. Whilst some of these forms have banding patterns resembling the standard profile of liquid culture grown *S.lacrymans*, some are very different. In particular it has not proved possible to obtain any profile for spore extracts, possibly because protein material from spores does not extract into the SDS containing extraction buffer used in these studies. Strand material has been found to possess only one major band, of molecular weight around 60,000 daltons, in comparison with the extracts of liquid culture grown mycelial material that possess upwards of 30 bands in their profiles.

As well as being able to identify fungi directly from extracts of field material, in the form of extracts of biomass of the fungus, it would also be advantageous to be able to directly identify *S.lacrymans* in infected wood extracts. For this to be possible a first requirement would be that extracts of uninfected wood should not produce complex banding patterns when analysed by SDS-PAGE. Initial experiments carried out on sterile, freeze dried, macerated and ground-up blocks of pine or lime indicated the presence of only three protein bands in SDS extracts. Presumably this is due either to the low protein content of wood or the relative insolubility or non-extractability of wood components into SDS containing buffers.

In contrast to the results found for uninfected wood, samples of pine or lime blocks infected with *S.lacrymans* revealed a complex protein profile which, though bearing resemblances to that found for liquid culture grown *S.lacrymans*,was nevertheless somewhat different. Initial observations on field material, ie wood thought to be infected by *S.lacrymans*, indicates that direct identification using SDS-PAGE may be difficult since profiles obtained to date show little resemblance to standard material. However it should be noted that i) it is difficult to definitively state that a specific piece of wood contains mycelium of a particular organism, ii) such material is unlikely to contain a pure culture and the banding pattern produced by *S.lacrymans* may well be obscured by the presence of other resident organisms and iii) there may be an effect of wood species on the banding pattern obtained. Regarding this final point, in the two species of wood that we have infected experimentally *viz.* pine and lime, species had little effect of the banding patterns subsequently obtained.

The conclusion to be drawn from these results is that whilst *S.lacrymans* isolates give consistent SDS-PAGE profiles when grown under identical conditions, when *S.lacrymans* FPRL 12C was grown under different conditions, and in particular when it was grown on wood as the major nutrient source, somewhat different profiles were produced. Whether such profiles are consistent within a particular set of conditions is currently under investigation. Similarly it may well be that the different patterns found for different morphological forms of *S.lacrymans* are consistent within those forms, e.g. strand material may always give the same profile which may be rather different from the profile found for the standard preparation of *S.lacrymans* used in these studies. Thus, given an appropriate standard and guidelines for sample collection, it may be possible, for example, to identify material directly from the surface of infected wood without intervening isolation procedures. (A suitable standard in this case would be a sample of mycelia which was known to be *S.lacrymans* and which had been removed from the surface of a wood sample.) However whilst identification of *S.lacrymans* within infected wood may be possible from extracts of pure culture derived from laboratory infected wood, identification in a complex system, as would be expected in a field sample, seems less likely. These possibilities are under currently under investigation.

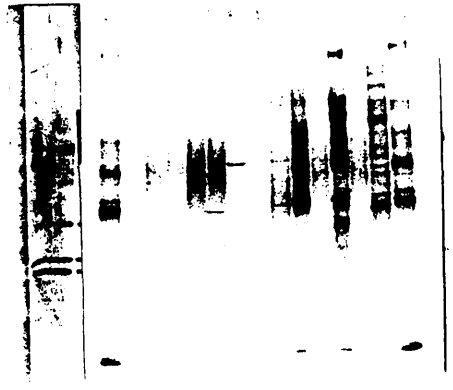
IMMUNOLOGICAL STUDIES ON *S.lacrymans*

In order to be able to identify *S.lacrymans* in the type of complex mixture which could exist in a sample of infected wood it is necessary to develop specific probes. This would avoid the need to first isolate the organism and then subject it to SDS-PAGE and might also allow detection of the organism when there are little or no visible signs of colonisation. Foremost amongst molecular probes are antibodies and the application of immunological technology to *S.lacrymans* has allowed the production of both polyclonal and monoclonal reagents. Simple immunisation of a rabbit with freeze dried

extracts of *S.lacrymans* FPRL 12C produced an antiserum, which, whilst it showed little specificity for this organism could nevertheless detect the fungus in wood when it was present at early stages of the decay process. Thus small pine blocks infected with *S.lacrymans*, but with weight losses of less than 5% (indicating a relatively early stage of degradation of the macromolecular material of the block), gave positive results in simple immunoassays such as dot blot assays and other enzyme immunoassays. Furthermore, western blot analysis on extracts of such blocks revealed a distinct pattern of antigen bands. Similar types of western blot analysis on extracts of liquid culture grown material indicated banding patterns which, whilst not identical to the wood grown material, were relatively similar. By contrast western blot analysis of other wood decay basidiomycetes indicated a very wide range of different profiles (Fig 3). Thus, as with SDS-PAGE, organisms can be readily identified on the basis of a macromolecular profile, in this case an antigen profile rather than a protein profile. Again, as with SDS-PAGE analysis, profiles for *S.lacrymans* and *S.himantioides* were similar whereas profiles for the other former members of the *Serpula* genus, *L.tignicola* and *L.pinastri* were very different.

Fig 3 Reaction of the *S.lacrymans* antiserum with a range of different fungal species.

Tracks 1 and 17 contained molecular weight markers, 2 and 16 the standard preparation of *S.lacrymans* and tracks 3 to 15 *D.concentrica*, *C.versicolor*, *D.quercina*, *P.placenta*, *P.gigantea*, *G.sepiarium*, *L.lepideus*, *L.pinastri*, *P.incrassata*, *A.xantha*, *P.panuoides*, *F.vaillantii* and *C.puteana* respectively.



Notable differences between the antigen and the protein profiles obtained in these studies relate to the appearance of the major components found in the two systems. Firstly, protein profiles consistently reveal upwards of 30 very well defined bands whereas fewer antigen components are found. Secondly in the blotting system used in these studies (a semi-dry method developed by Khyse-Anderson (1984), from the basic method of Towbin *et al* (1979)) the major antigen bands located for *S.lacrymans* were rather indistinct and did not directly relate to any of the major protein bands. Extensive efforts were made to increase the sharpness of the bands by the use of proteolytic inhibitors in extraction buffers, the cooling of all reagents to 4oC and a reduction in the time required for processing prior to application of the samples to the gels. None of these had a marked effect on the nature of the major antigens. It therefore seems probable that these antigens are particularly susceptible to post-translational processing in some way. A comparison of the major antigens in standard liquid culture material and in infected wood revealed similar banding patterns. However the molecular weights of the two major antigens extracted from infected wood were somewhat higher than those found in the liquid culture material. It seems possible therefore that the molecular processing event, which may well relate to some type of chain degradation, is more advanced in the liquid culture material than in material extracted from infected wood blocks.

As Fig 3 indicates the antiserum produced in this study showed relatively little specificity. It could be used for identification of pure culture material using western blotting techniques but its relevance to the direct detection and identification of field material was limited. However, since the SDS-PAGE profiles and the antigen profiles obtained for different decay organisms both appeared to be unique, the successful development of more specific antibody based molecular probes seemed a probability. To this end, and in common with other areas of mycology where specific organism detection is required (e.g. Mitchell 1988, Dewey *et al* 1989) it seemed that a possible method to produce reagents with the required degree of specificity would be to develop monoclonal antibodies. Using a simple immunisation schedule based on that used to produce polyclonal antisera to other decay fungi (Glancy *et al* 1989) mice hyperimmunised with *S.lacrymans* were prepared, and monoclonal antibodies produced, by simple modifications of the original method of Kohler and Milstein (1974). Whilst a number of relatively non-specific antibodies were produced by this technique some antibodies have been obtained that exhibit the required degree of specificity (Table 2). The possible use of such reagents as the basis of simple detection systems is currently under test.

Table 2 Cross reactivity of antibodies with a variety of fungal isolates

Antibody	Fungal Isolate									
(code no.)	1	2	3	4	5	6	7	8	9	10
7/22	-	-	-	-	-	-	-	-	+	+
7/180	-	-	+	+	-	-	-	+	-	+
7/465	+	+	+	+	-	-	+	-	+	+
7/704	-	-	-	-	-	-	-	-	-	+
7/705	-	-	-	-	-	-	-	-	-	+
7/38	-	-	-	-	+	-	-	-	-	+
7/26	-	+	-	+	-	-	-	+	-	+
11/18	+	-	-	+	-	-	+	-	-	+
11/64	-	-	-	-	+	+	+	+	-	+
11/55	+	+	-	+	+	+	+	+	-	+
11/48	+	+	+	+	+	+	+	+	+	+

+ indicates a positive reaction in an enzyme immunoassay test
- indicates a negative reaction in the immunoassay test

The following fungi were tested for cross reactivity with the 11 monoclonal antibodies listed above. 1: *C.puteana*, 2: *F.vaillantii*, 3: *L.pinastri*, 4: *P.gigantea*, 5: *D.concentrica*, 6: *C.picea*, 7: *P.panuoides*, 8: *D.quercina*, 9: *P.incrassata*, 10: *S.lacrymans*. Details of all isolates used can be found in Palfreyman *et al* (1990).

CONCLUSIONS

These studies demonstrate that analysis of the protein components of *S.lacrymans* either by SDS-PAGE or by use of antibody probes can be useful in the identification of the organism, that the nature of the major antigens detected when the organism grows on different substrates varies and that organism specific probes for *S.lacrymans* can be produced. As well as improving

detection and identification of *S.lacrymans* the techniques and reagents discussed in this paper will undoubtedly contribute to studies relating to numerous other aspects of the organism.

REFERENCES

Blum, H., Beier,H. and Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis, 8 93-99.

Cartwright, K.St.G. and Findlay, W.P.K. (1958). Decay of timber and its prevention. Forest Products Research Laboratory. Department of Scientific and Industrial Research. H.M.S.O., London, U.K..

Dewey, F.M., MacDonald, M.M. and Phillips, S.I. (1989). Development of monoclonal-antibody-ELISA, -dot-blot and -dip-stick immunoassays for *Humicola lanuginosa* in rice. J. gen. Microbiol., 135 361-374.

Glancy, H., Bruce, A., Button, D., Palfreyman, J.W. and King, B. (1989). Application of immunological methods to the analysis and detection of Letinus lepideus Fr.. International Research Group on Wood Preservation. Document No. IRG/WP/1422

Goodell, B.S. and Jellison, J. (1986). Detection of a brown rot fungus using serological assays. International Research Group on Wood Preservation. Document No. IRG/WP/1305.

Hansen, E.M., Brasier, C.M., Shaw, D.S. and Hamm, P.B. (1986). The taxonomic structure of Phytophthora megasperma: evidence for emerging biological species groups. Trans. Br. mycol. Soc., 87 557-573.

Jackman, P.J.H. (1985). Characterisation of microorganisms by electrophoretic protein patterns. In 'New methods for the detection and characterisation of microorganisms'. Edited by C.S. Gutteridge. Wiley, U.K..

Jeng, R.S. and Hubbes, M. (1983). Identification of aggresive and nonaggressive strains of *Cerastocystis ulmi* by polyacrylamide gel electrophoresis of intra-mycelial proteins. Mycotaxon, 17 445-455.

Kersters, K. (1985). Numerical methods in the classification of bacteria by protein electrophoresis. In 'Computer assisted bacterial systematics'. Edited by M. Goodfellow, D. Jones and F.G. Priest. Academic Press, U.K..

Khyse-Anderson, J. (1984). Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods, 10 203-210.

Kohler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256 495-497.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bactriophage T4. Nature, 227 680-685.

Marsden, H.S., Stow, N.D., Preston, V.G., Timbury, M.C. and Wilkie, N.M. (1978). Physical mapping of herpes simplex virus induced polypeptides. J. Virol., 28 624-642.

Mitchell, L.A. (1988). A sensitive dot immunoassay employing monoclonal antibodies for detection of *Sirococcus strobilinus* in spruce seed. Plant Disease, 72 664-667.

Palfreyman, J.W., Bruce, A., Button, D., Glancy, H., Vigrow, A. and King, B. (1987). Immunological methods for the detection and characterisation of wood decay basidiomycetes. In 'Biodeterioration VII', pp.709-713. Edited by D.R. Houghton, R.N. Smith and H.O.W. Eggins. Elsevier Applied Science, U.K..

Palfreyman, J.W., Vigrow, A., Button, D., Hegarty, B. and King, B. (1991). The use of molecular methods to identify wood decay organisms, the electrophoretic analysis of *Serpula lacrymans*. Wood Protection 1, 15 — 22.

Schmidt, O. and Kebernik, V. (1989). Characterisation and identification of the dry rot fungus *Serpula lacrymans* by polyacrylamide gel electrophoresis. Holzforschung, 43 195-198.

Seehann, G. and Hegarty, B.M. (1988). A bibliography of the dry rot fungus, *Serpula lacrymans*. International Research Group on Wood Preservation. Document No. IRG/WP/1337.

Tariq, V.N., Gutteridge, C.S. and Jeffries, P. (1985). Comparative studies of cultural and biochemical characteristics used for distinguishing species within *Sclerotinia*. Trans. Br. mycol. Soc., 84 381-397.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A., 76 4350-4354.

Vigrow, A., Button, D., Palfreyman, J.W., King, B. and Hegarty, B. (1989). Molecular studies on isolates of Serpula lacrymans. International Research Group on Wood Preservation. Document No. IRG/WP/1421.

Vigrow, A., Palfreyman, J.W. and King, B. (1990). On the identity of certain isolates of *Serpula lacrymans*. (Submitted for publication).

de Vries, O.M.H., Hoge, J.H.C. and Wessels, J.G.H. (1980). Regulation of the pattern of protein synthesis in *Schizophyllum commune* by the incompatibility genes. Developmental Biology, 74 22-36.

Molecular Studies on Wood Decay Basidiomycetes

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Introduction

The majority of papers presented in this workshop have concerned the application of molecular methods to fungal plant pathogens. These methods are increasingly being used in other areas of mycology where identification and detection of fungi is important. Studies using these methods have been reported in areas which are as diverse as clinical diagnosis and evaluation of foodstuffs. This report concerns the application of molecular methods to the identification and detection of wood decay basidiomycetes. Studies at Dundee Institute of Technology have concentrated on Lentinus lepideus (a major cause of failure of creosote treated timber) and Serpula lacrymans (the causative organism of dry rot). Results relating to the latter organism are presented in this paper.

Identification of S. lacrymans

The identification of S. lacrymans poses little problem to experts in fungal taxonomy. The fruit body of the organism is easily distinguished from other basidiomycetes, with the possible exception of S. himantioides, whilst in laboratory culture the organism shows a number of specific characteristics, e.g. an unusual heat sensitivity and low temperature growth optimum. However identification of S. lacrymans by less experienced persons is not easy and disputes occasionally arise, with major financial implications, around the identification of outbreaks of decay fungi. Since protein analysis has been used in other areas of mycology to aid the identification of fungi, the applicability of this type of analysis to S. lacrymans was investigated.

A group of 14 isolates of S. lacrymans from Europe, the U.S.A. and Australia were obtained for analysis, grown on 5% malt extract broth at 22°C, harvested, freeze dried and stored at -20°C. The dried material was then analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A consistent banding profile was obtained for a series of extracts from different subcultures of the standard organism (FPRL 12C). A comparison of the range of 14 S. lacrymans isolates with the standard profile for FPRL 12C indicated that most of them were identical. However, two isolates had profiles which, although similar to FPRL 12C, showed some differences. One of these organisms has now been identified as S. himantioides.

If SDS-PAGE is to be used for identification purposes, it is essential that other organisms in a similar environment must be easily distinguished from S. lacrymans on the basis of their protein profiles. A number (18) of other wood decay basidiomycetes were analysed by SDS-PAGE and all of them produced different profiles to both S. lacrymans and each other. These studies, now confirmed by graphical analysis of gel patterns, have indicated that identification of S. lacrymans by SDS-PAGE is feasible and the technique has been validated on a number of new, putative, isolates of S lacrymans which gave protein profiles similar to FPRL 12C.

Further studies have shown that when S. lacrymans FPRL 12C was grown on wood different profiles were produced compared with those consistently obtained when it was grown on malt extract. Whether such profiles are consistent within a particular set of conditions is currently under investigation. Different protein patterns were also found for different morphological

forms of S. lacrymans and this may be consistent within those forms, e.g. strand material may always give the same profile which may differ from that found for the standard preparation of S. lacrymans. Thus, given an appropriate standard and guidelines for sample collection, it may be possible to identify material taken directly from the surface of infected wood without intervening isolation procedures. Whether such methodology would also be appropriate to the identification of organisms within wood samples, where specific identification of an organism against a background of other organisms is required, is under investigation.

Immunological Studies on S. lacrymans

To facilitate identification of S. lacrymans in the type of complex mixture which could exist in a sample of infected wood, it was necessary to develop specific probes. Simple immunisation of a rabbit with freeze dried extracts of S. lacrymans FPRL 12C mycelium produced an immunological probe which although showing little specificity for the fungus, could nevertheless detect it in wood at early stages of decay. Small pine blocks infected with S. lacrymans, but with weight losses of less than 5% (indicating a relatively early stage of degradation of the macromolecular material of the block), gave positive results in simple immunoassays. Western blot analysis on extracts of such blocks revealed a distinct pattern of antigen bands. Furthermore Western blot analysis on extracts of liquid culture grown material indicated banding patterns which were relatively similar but had a few notable differences to the wood grown material. By contrast, Western blot analysis of other wood decay basidiomycetes indicated a very wide range of different profiles. Thus the use of immunological probes enables organisms to be readily identified on the basis of a macromolecular profile, in this case an antigen profile rather than a protein profile. Antigen profiles for S. lacrymans and S. himantioides were similar confirming earlier concusions from SDS-PAGE analysis.

The antiserum to S. lacrymans could be used for identification of pure culture material using Western blotting but its relevance to direct detection and identification of field material was limited. However, since the SDS-PAGE profiles and the antigen profiles obtained for different decay organisms both appeared to be unique, the successful development of more specific antibody based molecular probes seemed a probability. Monoclonal antibodies were developed using a simple immunisation schedule based on that used to produce polyclonal antisera to other decay fungi. Mice hyperimmunised with S. lacrymans were prepared and monoclonal antibodies produced by simple modifications of the original method of Kohler and Milstein. Whilst a number of non-specific antibodies were produced, some antibodies have been obtained with the required degree of specificity. The possible use of such reagents in simple detection systems is currently under test.

Conclusions

These studies demonstrated firstly that analysis of the protein components of S. lacrymans either by SDS-PAGE or by use of antibody probes can be useful in the identification of the organism, secondly that the nature of the major antigens detected when the organism grows on different substrates varies and, finally that organism-specific probes for S. lacrymans can be produced. As well as improving methodology for the detection and identification of S. lacrymans, the techniques and reagents discussed in this paper will undoubtedly contribute to other studies relating to this and other wood decay basidiomycetes.

THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Working Group I a Biological Problems (Flora)

The antigenic nature of *Serpula lacrymans*

by

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Paper prepared for the 22nd Annual Meeting

Kyoto, Japan

20-24 May 1991

IRG Secretariat
Box 5607
S-114 86 Stockholm
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29 March 1991

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SUMMARY

Antigenic proteins from a variety of isolates of Serpula lacrymans and other wood decay basidiomycetes were compared using Western blotting. Most isolates of S.lacrymans gave identical profiles while other organisms gave very different profiles. The major antigens of S.lacrymans were not associated with any easily identifiable protein species and their appearance as rather indistinct bands suggests that they are either readily degraded during the organisms life cycle or extensively processed. S.lacrymans grown in wood samples produced more distinct bands when tested by Western blotting and the bands produced were similar in both molecular weight and appearance to bands from extracts of actively growing regions of mycelial cultures. It is suggested that these bands may represent specific molecular forms of antigens associated with particular organism growth phases and further that immunological methods may be applicable to the detection and identification of S.lacrymans grown in either liquid (or agar) culture or in its natural substrate, viz. wood.

KEY WORDS : Serpula lacrymans, immunoblotting, growth phase antigens

INTRODUCTION

Recent studies have indicated that the molecular nature of the wood decay fungus, Serpula lacrymans, can be uniquely defined using the technique of sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Vigrow et al 1989, Schmidt and Kebernik 1989). Whilst this technique can provide a standard method for isolate identification it is complex and cannot be performed simply as a routine method. However since molecular differences between basidiomycetes have been identified it is of interest to investigate whether other molecular based techniques could be used for identification purposes.

The extensive use of immunological methods in other areas of mycology, for example in the identification of plant pathogens, e.g. Phytophthora spp. (Hansen et al 1986) and in medical diagnosis, e.g. Aspergillus infections (Hipp et al 1970), suggested that detection and identification of basidiomycetes such as S.lacrymans might be possible using immunological methods and recent reports of the application of polyclonal and monoclonal antibodies to studies of such organisms have confirmed this idea (Goodell and Jellison 1986, Palfreyman et al 1987, Srebotnik et al 1988).

Immunological methods offer a significant advantage over current methodology as it has been shown that they can be used to detect decay fungi directly in wood samples (Palfreyman et al 1988) though antigenic differences were reported between cultures grown in liquid media and those extracted from wood. Whilst this is consistent with the molecular behaviour of fungal microorganisms it potentially raises problems since it indicates that if more specific molecular probes to S.lacrymans are developed they may not react with the organism when grown on different substrates. In order to define the antigenic nature of S.lacrymans the range of different isolates which were used in previous studies (Vigrow et al 1989) have been investigated by Western blotting. Their antigenicity under different growth conditions has also been

studies. The results are presented in this paper.

MATERIALS AND METHODS

Antigens and antiserum

Fungal isolates used in this study have previously been described (Vigrow et al 1989) and samples used for SDS-PAGE and Western blotting were prepared as detailed in that paper. S.lacrymans FPRL 12C was prepared as an immunogen as decribed for Lentinus lepideus (Glancy et al 1989) and immunised into a New Zealand White rabbit in Freund's complete adjuvant (primary immunisation) followed by Freund's incomplete adjuvant (secondary immunisation). Both types of immunisation used 5mg of antigen dispersed in 2ml of phosphate buffered saline (PBS)/adjuvant emulsion and consisted of multiple sub-cutaneous injections.

Analysis of variation between morphological regions of S.lacrymans was carried out using a similarity index reported in Palfreyman et al (1990)

Electrophoresis and Immunoblotting

Extracts of fungal isolates were separated by SDS-PAGE using the method of Laemmli (1970). Gradient gels were used throughout (Vigrow et al 1989). The Western blotting of separated samples on to Immobilon (Millipore Ltd., U.K.) was achieved by using the Sartoblot semi-dry electrophoretic transfer system (Sartoblot II, Sartorius Ltd.) following the method of Towbin et al (1979) as modified by Khyse-Anderson (1984).The manufacturer's protocol was used throughout except that the blotting process was carried out at 0.8 mA/cm2 of gel for 30 min at 4oC followed by a further 1.2 mA/cm2 of gel for 30 min. To check that efficient transfer of antigens had occurred strips of Immobilon containing separated standard proteins were stained using the Indian ink method of Hancock and Tsang (1983). Gels to be analysed directly for protein were stained by the silver staining method of Blum et al (1987).

The Immobilon was washed x2 with PBS/0.05% Tween 20 after blotting. The low molecular weight standard protein (LMWt standards) tracks were cut off and stained as described above. The non-specific binding sites on the Immobilon were blocked for 1 hour at 4oC using PBS containing 0.5% Tween 20 and 10% new born calf serum (NCS) and stained by i) incubating overnight at 4oC with S.lacrymans antiserum diluted 1:1600 in PBS/ 0.05% Tween 20/ 5% NCS, ii) 5 washes with PBS/0.05% Tween 20, iii) incubation for 1h with horseradish peroxidase linked anti rabbit serum (1:200 in PBS/0.05% Tween 20/ 5% NCS) at room temperature, iv) 6 washes with PBS/0.05% Tween 20, v) 2 washes with PBS and vi) bound antibody detected with diaminobenzidine or diaminobenzidine enhanced with nickel chloride. When the latter detection system was used 2 additional washes with Tris 50mM pH 7.6 preceded the use of the chromogen. In both cases colour development was stopped with a final PBS wash prior to drying the Immobilon.

Preparation of other S.lacrymans extracts

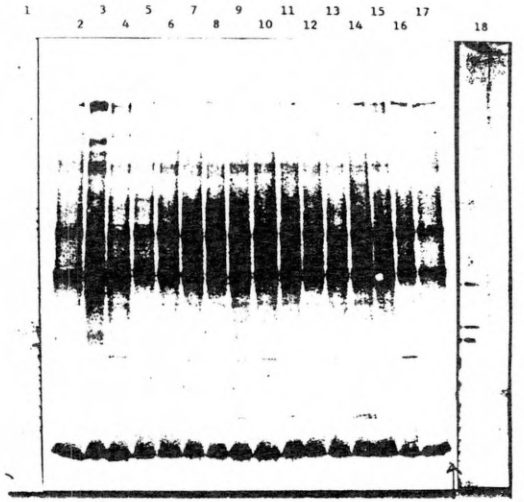
S.lacrymans decayed wood blocks were prepared as described in Palfreyman et al (1990) except that 1cm cube pine wood blocks were used in addition to lime blocks, and extracts prepared for SDS-PAGE and subsequent Western blotting as also described in that paper. 'Young' mycelia were prepared from agar grown S.lacrymans, only the peripheral 5mm of such cultures being harvested by carefully scraping of the mycelium from the agar surface. The 5mm region of mycelium around the innoculation core was harvested in a similar manner and was termed 'aged' mycelia. Both 'young' and 'aged' mycelia were freeze dried prior to further analysis.

RESULTS

The antigenic profiles obtained by Western blotting for 15 different isolates of S.lacrymans are shown in Fig.1. With the exception of BF-015B all isolates gave similar profiles. In all isolates two major antigens were found of molecular weight 51,000-54,500 daltons and 41,500-43,000 daltons and, as can be seen from Fig. 1, both of these antigens produced relatively diffuse bands. A variety of minor antigens were detected, the intensity and definition of some of these bands varied from experiment to experiment.

Fig 1.

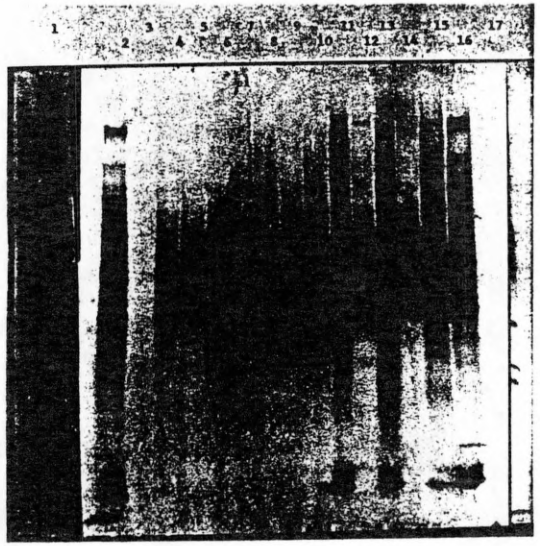
Western blot analysis of extracts of isolates of S.lacrymans. Tracks 1 and 18 represent Indian ink staining of molecular weight markers. Tracks 2 and 17 represent extracts of S.lacrymans FPRL 12C, tracks 3 to 16 represent the following isolates of S.lacrymans, BF-015B, BF-050, BF-072, BF-049, BF-046, BF-044, BF-025, BF-023, BF-018A, BF-17B, BF-07B, BF-03A, BF-01, and CMI 152233 respectively



A comparison of the antigenic profile of the type strain of S. lacrymans (FPRL 12C) with a range of other basidiomycete fungi which can be isolated from hard and softwoods of commercial importance is shown in Fig 2. All fungi tested gave patterns which were very different to that produced by S.lacrymans. The two major antigens of S.lacrymans were missing in most other organisms, with the exception of Poria incrassata and, possibly, Paxillus panuoides. However, a degree of cross reactivity of the antiserum was noted with all fungi except Daldina concentrica. This cross reactivity was particularly marked in some of the basidiomycetes which, like S. lacrymans, cause rot of softwoods in buildings, viz. Coniophora puteana, P.panuoides and P.incrassata. However, 2 other basidiomycetes which also cause rot of building softwoods showed little cross reactivity, viz. Amyloporia xantha and Fibrioporia vaillantii.

Fig 2.

Reaction of the S.lacrymans antiserum with a range of different fungal isolates. Tracks 1 and 17 represent molecular weight markers. Tracks 2 and 16 represent the standard profile of S.lacrymans FPRL 12C. Tracks 3 to 15 represent D.concentrica, Coriolus versicolor, Daedalea quercina, Postia placenta, Penipohora gigantea, Gloeophyllum sepiarium, L.lepideus, Serpula pinastri, P.incrassata, A.xantha, P.panuoides, F.vaillantii and C.puteana respectively.

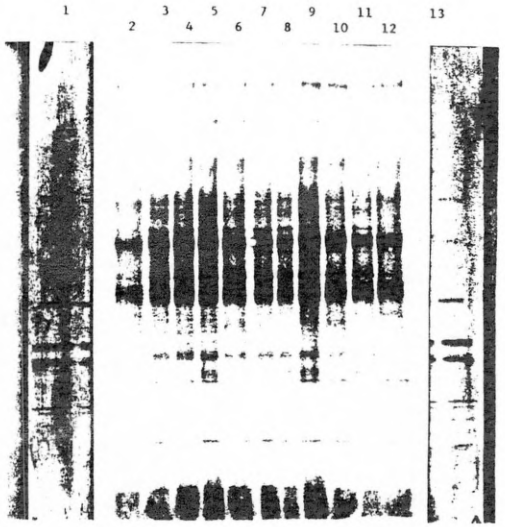


The results shown in Fig 1 and 2 indicate a diffuseness of the major bands associated with the antigenic material of most of the wood rotting basidiomycetes sampled. By contrast, the antigen patterns of S.pinastri, L.lepideus and G.sepiarium showed only one cross reacting protein displayed as a sharp band. The diffuseness of antigen bands displayed by the majority of fungal isolates is in contrast to both the silver stained SDS-PAGE profiles for these fungi (Vigrow et al 1989) and Indian ink staining of patterns of proteins transferred to Immobilon (data not shown) all of which gave sharp bands.

A number of procedures were tried to improve the sharpness of the bands detected by Western blotting, the most successful being either the use of inhibitors of proteolysis or the processing of samples at 4oC and their initial extraction being made directly into PBS/ boiling mix (2:1) at 4oC (Fig.3). The latter procedure was used for all subsequent gels. However, even using this methodology it was impossible to produce an antigenic profile which compared in sharpness to the protein profiles produced by silver or Indian ink staining.

Fig 3.

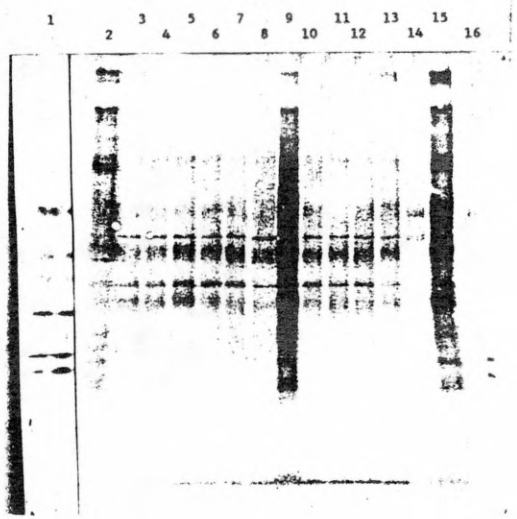
Effect of proteolytic inhibition on the antigenic profile of S.lacrymans FPRL 12C. Tracks 1 and 13 represent molecular weight markers. Tracks 2 and 12 are the standard preparation of FPRL 12C. Tracks 3 to 11 represent extracts of S.lacrymans treated or prepared using different conditions. Samples in tracks 4,6,8 and 10 were extracted at room temperature, those in tracks 3,5,7,9 and 11 were prepared at 4oC. Samples in tracks 7,8,9 and 10 were extracted directly into boiling mix and either boiled for 3 min prior to storage at -20oC (7,8) or stored immediately at -20oC (9,10). Samples in tracks 3,4,5 and 6 were extracted into a solution containing phenylmethylsulphonyl fluoride (PMSF) and either boiled prior to freezing (3,4) or frozen immediately after extraction (5,6). Samples in track 11 were kept at 4oC until storage by freezing.



To compare the antigenic banding pattern of liquid culture and wood grown material, 1 cm cube pine blocks were infected with S.lacrymans as described in the MATERIALS and METHODS . Blocks with weight losses between 0 and 24% were produced and detergent (SDS) soluble material extracted from the milled blocks and analysed by SDS-PAGE and Western Blotting. The results are shown in Fig 4. Undecayed wood showed very few bands in the stained gels and no bands in the Western blot. Silver stained bands obtained in the blocks showing decay were somewhat similar to the standard profile of S.lacrymans FPRL 12C and estimation of the similarity index for four of the decayed blocks (with weight losses in the range 1.56 to 20%) gave a mean value of 64.10 (±0.75). By contrast, the antigenic profiles of wood grown and liquid culture grown material were very different. It was particularly noticeable that the two major antigens which were present in the liquid culture were not present in the wood material. These were apparently replaced by two major antigens of molecular weight 57,000 and 43,000 daltons.

Fig 4.

Analysis of antigenic proteins of S.lacrymans FPRL 12C in infected pine sapwood. Tracks 1 and 16 represent molecular weight markers. Tracks 2,9 and 15 are extracts of FPRL 12C (standard preparation). Tracks 3-8 and 10-13 represent extracts from infected wood blocks with the following weight losses (3) 26.73%, (4) 24.51%, (5) 19.28%, (6) 15.44%, (7) 11.03%, (8) 8.29%, (10) 4.24%, (11) 1.02%, (12) 0.47%, (13) 0.0%. A sample of uninfected wood is shown in track 14

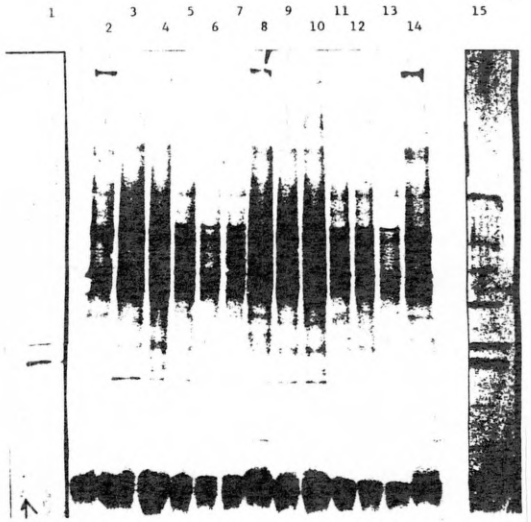


It was also noted that the major antigenic bands present in the decayed wood samples were very much sharper than those present in the liquid culture material.

Previous studies have shown that morphologically different structures of S.lacrymans produced different protein profiles when analysed by silver staining of SDS-PAGE gels (Vigrow et al 1989). Extracts of young and aged mycelia were compared in order to investigate the nature of the antigenic profiles of different regions of agar grown cultures of FPRL 12C. Some differences were seen in protein profiles between the 2 extracts (data not shown) but major differences were apparent when antigen profiles were compared (Fig.5). It was also noted that the pattern for the young mycelium was occasionally displayed as a component of the standard profile for S.lacrymans 12C (Fig 5), however the standard profile more clearly mirrored the pattern found in the aged mycelium. Overall it could be concluded that the antigen profile for the standard preparation of S.lacrymans 12C was a composite of the young and aged mycelial patterns. It should be noted that aged and young mycelia were harvested from agar cultures whereas the standard FPRL 12C preparation was cultured in liquid medium. To check whether the form of medium had any effect on the antigenic patterns a comparison was made between that for the standard liqiud cultured mycelium and composite mycelial material scraped from the whole of the surface growth on an agar plate. Such harvests gave identical antigenic profiles (Fig 6).

Fig 5.

Comparison of the antigenic profiles of young and aged mycelial extracts of S.lacrymans. Tracks 1 and 15 represent molecular weight markers. Tracks 2,8 and 14 are the standard preparation of S.lacrymans FPRL 12C. Tracks 11,12 and 13 are extracts of young mycelia from a first subculture of FPRL 12C, tracks 5,6 and 7 are similar extracts from material cultured some 6 months later. Tracks 9 and 10 are extracts of aged mycelia from the first subculture, tracks 3 and 4 represent similar material from the later culture.



Comparison of the results shown in Fig. 4 and Fig. 5 indicate that the antigen pattern for the young mycelium very closely resembled that of pine and lime sapwood grown mycelium. Confirmation of this observation is given in Fig. 7, with antigens from the 2 wood types producing similar patterns in Western blotting. These results confirm that the antigens found in young growing regions of S.lacrymans FPRL 12C and in pine and lime sapwood (0-20% and 0-23% weight losses respectively) are similar in molecular weight and possibly also in identity. In further support of this it was found that a comparison of the silver stained profiles of the young mycelial regions of S.lacrymans with the 4 blocks noted above (weight loss in range 1.56 to 20.0%) gave a mean smilarity index of 72.62 $\pm$ 2.88, a value which is significantly higher ($p>0.01$) than that found when the wood material was compared with the standard FPRL 12C preparation.

Fig 6.

Effect of different culture media on the antigenic profile of S.lacrymans FPRL 12C. Tracks 1 and 17 are molecular weight markers. Tracks 2,9 and 16 are the standard preparation of S.lacrymans FPRL 12C. Tracks 10-15 represent material harvested from liquid culture (3 subcultures at different days of uplift), tracks 3-8 represent solid culture material (3 subcultures at different days of uplift).

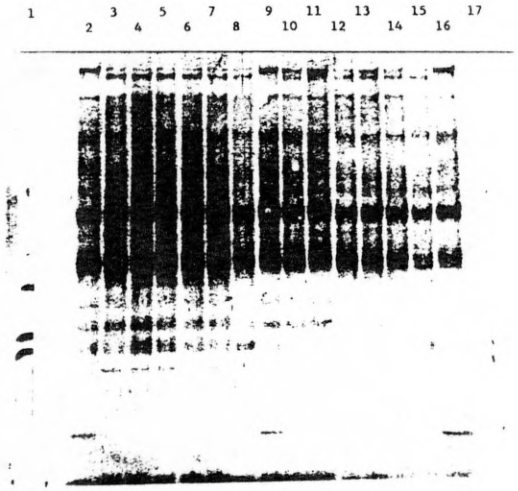
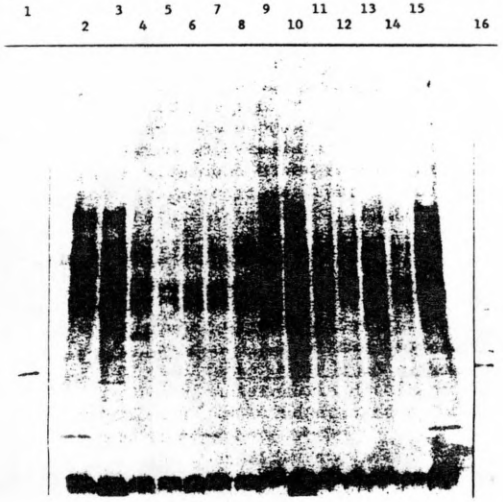


Fig 7.

Comparison of antigenic profiles for young mycelia and S.lacrymans infected pine and lime sapwood. Tracks 1 and 16 represent molecular weight markers. Tracks 2 and 15 are the standard preparation of S.lacrymans. Tracks 3 and 10 represent extracts of young S.lacrymans mycelia. Tracks 4,6,11,12,13 and 14 represent extracts of infected pine blocks with weight losses of 45.2%, 39.84%, 19.28%, 20.66%, 1.02% and 2.77% respectively. Tracks 5,7,8 and 9 represent infected lime blocks with weight losses of 61.99%, 54.91%, 23.27 and 0.0% respectively.



DISCUSSION

Previous studies have indicated that protein profiles obtained by SDS-PAGE for different genera and species of wood decay fungi show substantial and reproducible differences (Vigrow et al 1989, Smith and Kebernik 1989). Such studies together with information from other areas of mycology indicate that antigenic differences between wood decay fungi will also occur and these have been demonstrated using an antiserum against L.lepideus (Glancy et al 1989). Exploitation of these differences would allow the wide range of immunological techniques to be applied to organism identification as they are already being applied to features of the decay process (Srebotnik et al 1988, Palfreyman et al 1988).

Using a simple immunisation schedule we have produced an antiserum which, whilst it shows little specificity towards the immunogen used, S.lacrymans, nevertheless reveals considerable antigenic variation between a range of fungi. The number of molecular species identified with this antiserum is somewhat less than the number identified by silver staining of polyacrylamide gels. However there is sufficient difference between organisms to propose the use of immunodetection systems as aids to organism identification. Indeed, using the Western blotting system described, it is probable that identification could be achieved even with the present, non-specific antiserum. Visual observation of banding patterns may be sufficient for identification purposes but it has been shown that it is possible to base identification methods on computerised data analysis of banding patterns of silver stained gels. These methods of numerical taxonomy use relatively sophisticated scanning and computerised statistical methods (Jackman et al (1983) and Kersters (1985)). Such systems have not yet been applied to wood decay organisms though it seems probable they could be used to analyse both stained gels and Western blot patterns if experimental conditions required their use.

Two major bands were routinely detected by the Western blotting of isolates of standard preparations of S.lacrymans. These bands, whilst they represent major antigens, are not well defined in silver stained SDS-PAGE profiles. Whilst the majority of proteins give very sharp bands on silver stained gels these two major antigens of S.lacrymans appeared as indistinct, i.e. badly defined, bands. Rigorous precautions to prevent protein degradation during the period between extraction and separation on an SDS-PAGE gel had little effcet on the nature of these bands. Furthermore, Indian ink staining of transferred proteins gave sharp banding patterns indicating that the indistinct nature of the major antigens was unlikely to be associated with degradation during the blotting procedure. It therefore seems probable that these antigens either represent processed forms of specific proteins, e.g. glycoproteins, or they are partial breakdown products of particular proteins. In either case, their visual absence on silver stained gels emphasises their high antigenicity. Alternatively they may be particularly resistant to staining by the method of Blum et al (1987). Experience with some other antisera that have been raised against different wood decay fungi indicates that the appearance of indistinct bands on Western blotting is not restricted to S.lacrymans. Interestingly, some other decay organisms which cross reacted with the S.lacrymans antiserum appeared to show a greater range of cross reacting proteins than the original immunogen.

It has been established for a number of years that various protein components of the mycelial phase of a fungus can alter with substrate, e.g. the production of specific digestive enzymes depends upon the growth substrate (Highley 1973). It was therefore of interest to determine if the major antigens present in liquid culture were also present when the fungus was grown in a wood substrate. Previous studies have shown that when S.lacrymans is grown in small lime blocks a number of differences are seen in SDS-PAGE protein profiles (Palfreyman et al 1990). This current paper shows differences

between antigenic profiles of mycelia grown in the two systems. Furthermore, the banding pattern found for wood extracted material was very much sharper than that found for the liquid culture material. The production of new antigens in infected wood suggests that these might represent digestive enzymes despite the fact that the immunogen used for antiserum production consisted of washed mycelium. However the similarity of the infected wood antigens to those detected in young mycelium indicates that these ahtigens are associated with the growth phase of the organism rather than the substrate utilised. Further experiments are underway to determine if these antigens are indeed associated with the phase of growth of the organism and to determine if other such antigens can be found for different decay organisms. Preliminary results with S.lacrymans isolate BF-015B, which is thought to be Serpula himantioides (Schmidt and Kerbernik 1989), indicate that antigenic profiles of young and aged mycelia of this organism are different (data not shown). If growth phase antigens exist then it may be that immunological methods will have the potential to determine organism status. Since such antigens appear to be present in infected wood, it may be possible to devise systems which can give information on the effectiveness, for example, of biocide treatments.

It will be noted that two major antigens are detected in both aged mycelia and young mycelia/infected wood extracts. These antigens may represent totally different molecular species. Alternatively the indistinct bands found in the aged mycelia may represent processed forms of the antigens present in the colonising mycelia. This hypothesis is currently under test and, if found to be correct, would indicate that the antigens detected do not simply represent the appearance of inducible enzymes, such as cellulases. It may be that the proteins detected immunologically may be glycoproteins, since these are known to be particularly antigenic (Gander 1974). In addition the molecular weight of particular glycoproteins can alter vary greatly during processing (Spear 1976,Haarr and Marsden 1981) consistent with the data found in this experiment.

REFERENCES

1. Gander, J.E. (1974). Fungal cell wall glycoproteins and peptido-polysaccharides. Ann. Rev. Microbiology 28, 103-119.

2. Glancy, H., Bruce, A., Button, D., Palfreyman, J.W. & King, B. (1989). Application of immunological methods to the analysis and detection of Lentinus lepideus Fr.. *International Research Group on Wood Preservation*. Document No. IRG/WP/ 1422.

3. Goodell, B.S. & Jellison, J. (1986). Detection of a brown rot fungus using serological assays. *International Research Group on Wood Preservation*. Document No. IRG/WP/ 1305.

4. Haarr, L. and Marsden, H.S. (1981). Two-dimensional gel analysis of HSV type 1-induced polypeptides and glycoprotein processing. J. gen. Virol. 52, 77-92.

5. Hancock, K. and Tsang, V.C.W. (1983). India ink staining of protein on nitrocellulose paper. Anal. Biochem. 133, 157-162.

6. Hansen, E.M., Brasier, C.M, Shaw, D.S. and Hamm, P.B. (1986). The taxonomic structure of Phytophthora megasperma: evidence for emerging biological species groups. Trans. Br. mycol. Soc., 87, 557-573.

7. Highley, T.L. (1973). Influence of carbon source on cellulase activity of white rot and brown rot fungi. Wood Fiber 5, 50-58.

8. Hipp, S.S, Berns, D.S, Tompkins, V. and Buckley, H.R. (1970). Latex slide agglutination test for Aspergillus antibodies. Sabouraudia 8, 237-241.

9. Hornung, U. and Jennings, D.H. (1981) Light and electron microscopical observations of surface mycelium of Serpula lacrymans: stages of growth and hyphal nomenclature. Nova Hedwigia 30, 101-126.

10. Jackman, P.J.H., Feltham, R.K.A. and Sneath, P.H.A. (1983). A program in BASIC for numerical taxonomy of micro-organisms based on electrophoretic protein patterns. Microbios. Lett. 23, 87-98.

11. Kersters, K. (1985). Numerical methods in the classification of bacteri by protein electrophoresis. In 'Computer assisted bacterial systematics' pp 337-368. Eds. Goodfellow, M., Jones, D., and Priest, F.G., Academic Press, U.K..

12. Kyhse-Anderson, J. (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. biochem. biophys. Methods 10, 203-210.

13. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227, 680-685.

14. Nobles, M.K. (1965). Identification of cultures of wood inhabiting hymenomycetes. Canadian J. Bot. 43. 1097-1139.

15. Palfreyman, J.W., Bruce, A., Button, D., Glancy, H., Vigrow, A. and King, B. (1987). Immunological methods for the detection and characterisation of wood decay basidiomycetes. In: Biodeterioration 7. Houghton, D.R., Smith, R.N. & Eggins, H.O.W., Eds., Elsevier Science Publishers Ltd, U.K.. 709-713.

16. Palfreyman, J.W., Glancy, H., Button, D., Vigrow, A., Score, A. & King, B. (1988). Use of immunoblotting for the analysis of wood decay basidiomycetes. *International Reseach Group on Wood Preservation*. Document No. IRG/WP 2307.

17. Palfreyman, J.W., Vigrow, A., Button, D., Hegarty, B. and King, B. (1990). (Submitted for publication).

18. Schmidt, O. & Kebernik, V. (1989). Characterisation and identification of the dry rot fungus Serpula lacrymans by polyacrylamide gel electrophoresis. Holzforschung. 43. 195-198.

19. Spear, P.G. (1976) Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their protein products in type-1 infected cells. J. Virol. 17, 991-1008.

20. Srebotnik, E., Messner, K., Foisner, R. and Pettersson, B. (1988). Ultrastructural localization of ligninase of Phanerochaete chryosporium by immunogold labeling. Current Microbiology 16, 221-227.

21. Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A 76, 4350-4354.

22. Vigrow, A., Button, D., Palfreyman, J.W., King, B. & Hegarty, B. (1989). Molecular studies on isolates of Serpula lacrymans. *International Research Group on Wood Preservation*. Document No. IRG/WP/ 1421.

23. De Vries, O.M.H. and Wessels, J.G.H. (1984) Pattern of polypeptide synthesis in non-fruiting monokaryons and a fruiting dikaryon of Schizophyllum commune. J. gen. Microbilogy 130, 145-154.